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(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

**0 393 690**  
**A1**

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 90107493.0

(51) Int. Cl.<sup>5</sup>: **C12N 15/52, C12N 9/00,**  
**C12P 23/00, C12P 9/00,**  
**C12P 5/00**

(22) Date of filing: 20.04.90

The applicant has filed a statement in accordance with Rule 28 (4) EPC (issue of a sample only to an expert). Accession number(s) of the deposit(s): FERM BP-2377.

(30) Priority: 21.04.89 JP 103078/89  
05.03.90 JP 53255/90

(43) Date of publication of application:  
24.10.90 Bulletin 90/43

(84) Designated Contracting States:  
DE ES FR GB IT NL

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(54) DNA sequences useful for the synthesis of carotenoids.

(57) Disclosed are DNA sequences which are useful for the synthesis of carotenoids such as lycopene,  $\beta$ -carotene, zeaxanthin or zeaxanthin-diglucoside, that is, DNA sequences encoding carotenoid biosynthesis enzymes. These DNA sequences are the sequences ① - ⑥, respectively, shown in the specification.

Also disclosed is a process for producing a carotenoid compound which is selected from the group consisting of prephytoene pyrophosphate, phytoene, lycopene,  $\beta$ -carotene, zeaxanthin and zeaxanthin-diglucoside, which comprises transforming a host with at least one of the DNA sequences ① - ⑥ described above and culturing the transformant.

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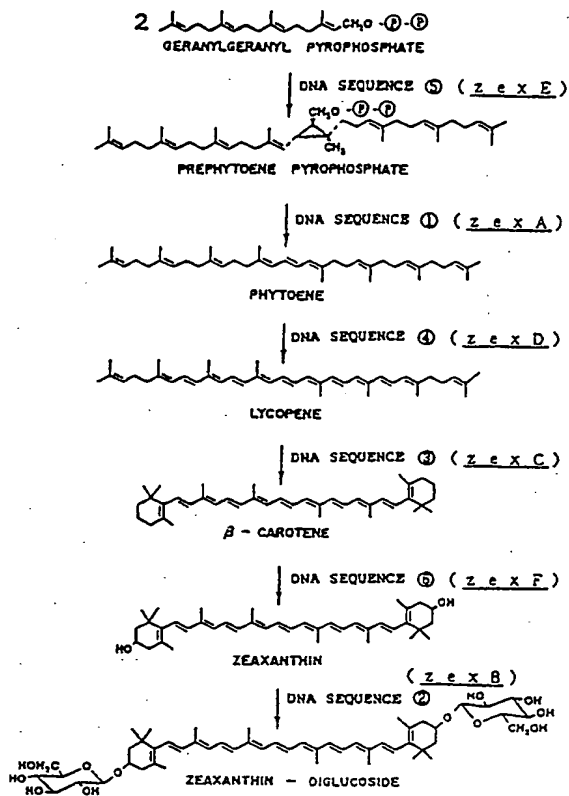


FIG. 8

## DNA SEQUENCES USEFUL FOR THE SYNTHESIS OF CAROTENOIDS

## BACKGROUND OF THE INVENTION

5 Field of the Art

The present invention relates to DNA sequences which are useful for the synthesis of carotenoids such as lycopene,  $\beta$ -carotene, zeaxanthin or zeaxanthin-digluconide.

The present invention also relates to processes for producing such carotenoid compounds.

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Related Art

Carotenoids are distributed widely in green plants. They are yellow-orange-red lipids which are also  
 15 present in some mold, yeast and so forth, and have recently received increased attention as natural coloring materials for foods. Among these carotenoids,  $\beta$ -carotene is a typical one, which is used as a coloring materials and as a precursor of vitamin A in mammals as well. It is also examined for its use as a component for preventing cancer [see, for example, SHOKUHIN TO KAIHATSU (Foods and Development), 24, 61-65 (1989)]. Carotenoids such as  $\beta$ -carotene are widely distributed in green plants, so that the plant  
 20 tissue culture has been examined for the development of a method for producing carotenoids in a large amount which is free from the influence of natural environment [see, for example, Plant Cell Physiol., 12, 525-531 (1971)]. The examination has been also made for detecting a microorganism such as mold, yeast or green algae which is originally high carotenoid productive and for producing carotenoids in a large amount with use of such microorganism (see, for example, The Abstract of Reports in the Annual Meeting  
 25 of NIPPON HAKKO KOGAKU-KAI of 1988, page 139). However, neither of these methods are successful at present in producing  $\beta$ -carotene at a good productivity which exceeds the synthetic method in commercial production of  $\beta$ -carotene. It would be very useful to obtain a gene group which participates in the biosynthesis of carotenoids, because it will be possible to produce carotenoids in a large amount by introducing a gene group which has been reconstructed to express proper genes in the gene group in a  
 30 large amount, into an appropriate host such as a plant tissue culture cell, a mold, an yeast or the like which originally produces carotenoids. Such a development in technology has possibilities for finding a method of producing  $\beta$ -carotene superior to the synthetic method and a method of producing useful carotenoids other than  $\beta$ -carotene in a large amount.

Furthermore, the synthesis of carotenoids in a cell or an organ which produces no carotenoid will be  
 35 possible by obtaining the gene group participating in the biosynthesis of carotenoids, which will add new values to organisms. For example, several reports have recently been made with reference to creating flower colors which cannot be found in nature by using genetic manipulation in flowering plants [see, for example, Nature, 330, 677-678 (1987)]. The color of flowers is developed by pigments such as anthocyanine or carotenoids. Anthocyanine is responsible for flower colors in the spectrum of red-violet-blue,  
 40 and carotenoids are responsible for flower colors in the spectrum of yellow-orange-red. The gene of the enzyme for synthesizing anthocyanine has been elucidated, and the aforementioned reports for creating a new flower color are those referring to anthocyanine. On the other hand, there are many flowering plants having no bright yellow flower due to no function of synthesizing carotenoids in petal (e.g. petunia, saintpaulia (african violet), cyclamen, Primula malacoides, etc.). If suitable genes having been reconstructed  
 45 so as to be expressed in petal in a gene group referring to the biosynthesis of carotenoids are introduced into these flowering plants, the flowering plants having yellow flowers will be created successfully.

However, enzymes for synthesizing carotenoids or genes coding for them have been scarcely elucidated at present. The nucleotide sequence of the gene group participating in the biosynthesis of a kind of carotenoids has been elucidated lately only in a photosynthetic bacterium *Rhodobacter capsulatus* [Mol.  
 50 Gen. Genet., 216, 254-268 (1989)]. But this bacterium synthesizes the acyclic xanthophyll spheroidene via neurosporene without cyclization and thus cannot synthesize general carotenoids such as lycopene,  $\beta$ -carotene and zeaxanthin.

There are prior arts with reference to yellow pigments or carotenoids of *Erwinia* species disclosed in J. Bacteriol., 168, 607-612 (1986), J. Bacteriol., 170, 4675-4680 (1988) and J. Gen. Microbiol., 130, 1623-1631 (1984). The first one of these references discloses the cloning of a gene cluster coding for yellow pigment

synthesis from Erwinia herbicola Eho 10 ATCC 39368 as a 12.4 kilobase pair (kb) fragment. In this connection, there is no illustration of the nucleotide sequence of the 12.4 kb fragment. The second literature discloses the yellow pigment synthesized by the cloned gene cluster, which is indicated to belong to carotenoids by the analysis of its UV-visible spectrum. The last literature indicates that the gene participating in the production of a yellow pigment is present in a 260 kb large plasmid contained in Erwinia uredovora 20D3 ATCC 19321 from the observation that the yellow pigment is not produced on curing the large plasmid, and further discloses that the pigment belongs to carotenoids from the analysis of its UV-visible spectrum.

However, the chemical structures of carotenoids produced by the Erwinia species or of its metabolic intermediates, enzymes participating in the synthesis of them or the nucleotide sequence of the genes encoding these enzymes remain unknown at present.

## DISCLOSURE OF THE INVENTION

### Outline of the Invention

The object of the present invention is to provide DNA sequences which are useful for the synthesis of carotenoids such as lycopene,  $\beta$ -carotene, zeaxanthin or zeaxanthin-diglucoside, that is DNA sequences encoding carotenoid biosynthesis enzymes.

In other words, the DNA sequences useful for the synthesis of carotenoids according to the present invention are the DNA sequences ① - ⑥ described in the following (1) - (6).

(1) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting prephytoene pyrophosphate into phytoene and whose amino acid sequence corresponds substantially to the amino acid sequence from A to B shown in Figs. 1-(a) and (b) (DNA sequence ①);

(2) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting zeaxanthin into zeaxanthin-diglucoside and whose amino acid sequence corresponds substantially to the amino acid sequence from C to D shown in Figs. 2-(a) and (b) (DNA sequence ②);

(3) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting lycopene into  $\beta$ -carotene and whose amino acid sequence corresponds substantially to the amino acid sequence from E to F shown in Figs. 3-(a) and (b) (DNA sequence ③);

(4) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting phytoene into lycopene and whose amino acid sequence corresponds substantially to the amino acid sequence from G to H shown in Figs. 4-(a), (b) and (c) (DNA sequence ④);

(5) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting geranylgeranyl pyrophosphate into prephytoene pyrophosphate and whose amino acid sequence corresponds substantially to the amino acid sequence from I to J shown in Figs. 5-(a) and (b) (DNA sequence ⑤); and

(6) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting  $\beta$ -carotene into zeaxanthin and whose amino acid sequence corresponds substantially to the amino acid sequence from K to L shown in Fig. 6 (DNA sequence ⑥).

Another object of the present invention is to provide processes for producing carotenoid compounds.

More specifically, the present invention also provides a process for producing a carotenoid compound which is related from the group consisting of prephytoene pyrophosphate, phytoene, lycopene,  $\beta$ -carotene, zeaxanthin and zeaxanthin-diglucoside, which comprises transforming a host with at least one of DNA sequences ① - ⑥ described above and culturing the transformant.

### Effect of the Invention

The successful acquirement of the gene group (gene group encoding the biosynthetic enzymes of carotenoids) useful for the synthesis of carotenoids such as lycopene,  $\beta$ -carotene, zeaxanthin, zeaxanthin-diglucoside or the like according to the present invention has made it possible to produce useful carotenoids in large amounts, for example, by creating a plasmid in which the gene(s) can be expressed in a large amount and employing an appropriate plant tissue culture cell, a microorganism or the like transformed with the plasmid. The success in acquiring the gene group useful for the synthesis of

carotenoids such as lycopene,  $\beta$ -carotene, zeaxanthin, zeaxanthin-diglucoside or the like according to the present invention has made it possible to synthesize carotenoids in cells or organs which produce no carotenoid by creating a plasmid in which the gene(s) can be expressed in a target cell or organ and transforming a suitable host with this plasmid.

### DETAILED DESCRIPTION OF THE INVENTION

The DNA sequences according to the present invention are the aforementioned DNA sequences ① - ⑥, that is, genes encoding the polypeptides of respective enzymes which participate in the biosynthesis reaction of carotenoids, in particular, for example, such polypeptides in Erwinia uredovora 20D3 ATCC 19321.

A variety of gene groups containing the DNA sequences of a combination of a plurality of sequences among these DNA sequences ① - ⑥ can be expressed in a microorganism, a plant or the like to afford them the biosynthesis ability of carotenoids such as lycopene,  $\beta$ -carotene, zeaxanthin, zeaxanthin-diglucoside or the like. The respective DNA sequences constructing the gene group may be present on a DNA strand or on different DNA strands individually, or optionally, the respective DNA sequences may comprise a plurality of DNA sequences present on a DNA strand and a DNA sequence present on another DNA strand.

The aforementioned gene group encode the polypeptides of a plurality of enzymes participating in the production of carotenoids. A recombinant DNA is created by incorporating the gene group into a proper vector and then introduced into a suitable host to create a transformant, which is cultured to produce mainly in the transformant a plurality of enzymes participating in the formation reaction of carotenoids and to conduct the biosynthesis of carotenoids in the transformant by these enzymes.

The DNA sequence shown in Fig. 7-(a) to (g), which is an example according to the present invention, is acquired from Erwinia uredovora 20D3 ATCC 19321 and thus exhibits, as illustrated in the experimental example below, no homology in the DNA-DNA hybridization with the DNA strand containing the gene group for synthesizing the yellow pigment of Erwinia herbicola Eho 10 ATCC 39368 (see Related Art described above).

### DNA Sequences encoding the polypeptide of each enzyme

The DNA sequences of the present invention are the DNA sequences ① - ⑥ (or the DNA strands ① - ⑥), respectively. Each of the DNA sequences contains a nucleotide sequence encoding the polypeptide whose amino acid sequence corresponds substantially to such an amino acid sequence as in the aforementioned specific regions in Figs. 1 - 6 (for example, from A to B in Fig. 1). In this connection the term "DNA sequence" means a polydeoxyribonucleic acid sequence having a length. In the present invention, the "DNA sequence" is defined by an amino acid sequence of a polypeptide which is encoded by the DNA sequence and has a definite length as described above, so that each DNA sequence has also a definite length. However, the DNA sequence contains a gene encoding each enzyme and is useful for biotechnological production of the polypeptide, and such biotechnological production cannot be performed by only the DNA sequence having a definite length but can be performed in the state where other DNA sequence with a proper length is linked to the 5'-upstream and/or the 3'-downstream of the DNA sequence. Therefore, the term "DNA sequence" in the present invention includes, in addition to those having a definite length (for example, the length in the region of A - B in the corresponding amino acid sequence of Fig. 1), those in the form of a linear DNA strand or a circular DNA strand containing the DNA sequence having a definite length as a member.

One of the typical forms of each DNA sequence according to the present invention is a form of a plasmid which comprises the DNA sequence as a part of a member or a form in which the plasmid is present in a host such as E. coli. The plasmid as one of the preferable existing forms of each DNA sequence according to the present invention is a conjunction of the DNA sequence according to the present invention as a passenger or a foreign gene, a replicable plasmid vector present stably in a host and a promoter (containing ribosome-binding sites in the case of a procaryote). As the plasmid vector and the promoter, an appropriate combination of those which are well-known can be used.

Polypeptides encoded by DNA sequences

As mentioned above, the DNA sequences according to the present invention are respectively specified by the amino acid sequences of the polypeptides encoded thereby. Each of these polypeptides is the one having an amino acid sequence which corresponds substantially to an amino acid sequence in a specific region as described above in Figs. 1 - 6 (for example, from A to B in Fig. 1). Here, in the six (A-B, C-D, E-F, G-H, I-J, K-L) polypeptides shown in Figs. 1-6 (i.e. six enzymes participating in the formation of carotenoids), some of the amino acids can be deleted or substituted or some amino acids can be added or inserted, etc., so long as each polypeptide has the aforementioned enzymatic activity in the relationship of a substrate and a converted substance (a product). This is indicated by the expression "whose amino acid sequence corresponds substantially to ..." in the claims. For example, each polypeptide that first amino acid (Met) has been deleted from each polypeptide shown in Figs. 1 - 6 is included in such deleted polypeptides.

The typical polypeptides having enzymatic activities, respectively, in the present invention are those in the specific regions in Figs. 1 - 6 described above, and the amino acid sequences of these polypeptides have not been known.

Nucleotide sequences of DNA sequences

The DNA sequences encoding the respective enzymes are those having the nucleotide sequences in the aforementioned specific regions in Figs. 1 - 6 (for example, A-B in Fig. 1) or degenerative isomers thereof, or those having the nucleotide sequences corresponding to the aforementioned alteration of the amino acid sequence of respective enzymes or degenerative isomers thereof. The term "degenerative isomer" means DNA sequence which is different only in degenerative codon and can code for the same polypeptide. The preferred embodiments of the DNA sequences according to the present invention are those having at least one stop codon (such as TAA) at the 3'-terminal. The 5'-upstream and/or the 3'-downstream of the DNA sequences according to the present invention may further have a DNA sequence with a certain length as a non-translation region (the initial portion of the 3'-downstream being usually a stop codon such as TAA).

Gene group used for the synthesis of carotenoids

The gene group (the gene cluster in some case) used for the synthesis of carotenoids comprises a plurality of the aforementioned DNA sequences ① - ⑥, whose typical examples are illustrated in the following (1) - (4). Each gene group encodes a plurality of polypeptides of respective enzymes and these enzymes participate in the production reaction of carotenoids to produce them from their substrates.

(1) Gene group used for the synthesis of lycopene

The gene group used for the synthesis of lycopene which is a red carotenoid is DNA sequence comprising the aforementioned DNA sequences ①, ④ and ⑤, and such a gene group includes the one in which respective DNA sequences are present on one DNA strand or on different DNA strands separately or the one which is constructed by the combination of the aforementioned ones according to necessities.

In the case that a plurality of DNA sequences are present on one DNA strand, the arrangement order and direction of the aforementioned DNA sequences ①, ④ and ⑤ may be optional provided that the genetic information is capable of expression, that is to say respective genes in a host are in a state of being transcribed and translated appropriately.

The biosynthetic pathway of lycopene in *E. coli* is explained as follows: geranylgeranyl pyrophosphate which is a substrate originally present in *E. coli* is converted into prephytoene pyrophosphate by the enzyme encoded by the DNA sequence ⑤, the prephytoene pyrophosphate is then converted into phytoene by the enzyme encoded by the DNA sequence ①, and the phytoene is further converted into lycopene by the enzyme encoded by the DNA sequence ④ (see Fig. 8).

Lycopene is a carotene whose color is red. Lycopene is a red pigment which is present in a large amount in the fruits of water melon or tomato and has high safety for food. In this connection, the lycopene which was synthesized by the DNA sequences according to the present invention in the experimental



example described below had the same stereochemistry as lycopene present in these plants.

One of the typical existing forms of the gene group of the present invention is a form of a plasmid which comprises the respective DNA sequences containing a stop codon as a member or a form in which the plasmid is present in a host such as *E. coli*. The plasmid which is one of the preferred existing forms of the gene group according to the present invention comprises a gene group as a passenger or a foreign gene, a replicable plasmid vector present stably in a host and a promoter (containing ribosome-binding sites in the case of a procaryote). As the promoter, in procaryotes such as *E. coli* or *Zymomonas* species a promoter which is common to respective DNA sequences can be used, or alternatively respective promoters can be used to the respective DNA sequences. In the case of eucaryotes such as yeast or plant, respective promoters are preferably used to respective DNA sequences.

One of the preferred existing forms of the DNA sequences are described above in the explanation of the DNA sequences ① - ⑥.

#### 15 (2) Gene group used for the synthesis of $\beta$ -carotene

The gene group used for the synthesis of  $\beta$ -carotene which is one of yellow-orange carotenoids is a DNA sequence comprising the aforementioned DNA sequences ①, ③, ④ and ⑤. In other words, the gene group used for the synthesis of  $\beta$ -carotene is formed by adding the DNA sequence ③ to a DNA sequence used for the synthesis of lycopene comprising the DNA sequences and ①, ④, and ⑤. The gene group includes the one in which the respective DNA sequences constructing the gene group may be present on one DNA strand or on different DNA strands individually, or the one which is constructed by the combination of the aforementioned ones according to necessities.

In the case that a plurality of DNA sequences are present on one DNA strand, the arrangement order and direction of the aforementioned DNA sequences ①, ③, ④ and ⑤ may be optional provided that the genetic information is capable of expression, that is to say respective genes in a host are in a state of being transcribed and translated appropriately.

The biosynthetic pathway of  $\beta$ -carotene in *E. coli* is explained as follows: geranylgeranyl pyrophosphate which is a substrate originally present in *E. coli* is converted into prephytoene pyrophosphate by the enzyme encoded by the DNA sequence ⑤, the prephytoene pyrophosphate is converted into phytoene by the enzyme encoded by the DNA sequence ①, the phytoene is further converted into lycopene by the enzyme encoded by the DNA sequence ④, and the lycopene is further converted into  $\beta$ -carotene by the enzyme encoded by the DNA sequence ③, (see Fig. 8).

$\beta$ -carotene is a typical carotene whose color is in the spectrum ranging from yellow to orange, and it is an orange pigment which is present in a large amount in the roots of carrot or green leaves of plants and has high safety for food. The utility of  $\beta$ -carotene has already been described in the explanation of related art. In this connection, the  $\beta$ -carotene which was synthesized by the DNA sequence according to the present invention in the experimental example described below had the same stereochemistry as  $\beta$ -carotene present in the roots of carrot or green leaves of plants.

One of the typical existing forms of the gene group and the individual DNA sequences are the same as defined in (1).

#### 45 (3) Gene group used for the synthesis of zeaxanthin

The gene group used for the synthesis of zeaxanthin which is one of yellow-orange carotenoids is a DNA sequence comprising the aforementioned DNA sequences ①, ③, ④, ⑤ and ⑥. In other words, the DNA sequence used for the synthesis of zeaxanthin is formed by adding the DNA sequence ⑥ to a DNA sequence used for the synthesis of  $\beta$ -carotene comprising the DNA sequences ①, ③, ④, ⑤. The gene group includes the one in which the respective DNA sequences constructing the gene group are present on one DNA strand or on different DNA strands individually, or the one which is constructed by the combination of the aforementioned ones according to necessities.

In the case that a plurality of DNA sequences are present on one DNA strand, the arrangement order and direction of the aforementioned DNA sequences ①, ③, ④, ⑤ and ⑥ may be optional provided that the genetic information is capable of expression, that is to say respective genes in a host are in a state of being transcribed and translated appropriately.

The biosynthetic pathway of zeaxanthin in *E. coli* is explained as follows: geranylgeranyl pyrophosphate which is a substrate originally present in *E. coli* is converted into prephytoene pyrophosphate by the

enzyme encoded by the DNA sequence ⑤ the prephytoene pyrophosphate is converted into phytoene by the enzyme encoded by the DNA sequence ①, the phytoene is then converted into lycopene by the enzyme encoded by the DNA sequence ④, and the lycopene is further converted into  $\beta$ -carotene by the enzyme encoded by the DNA sequence ③, and finally the  $\beta$ -carotene is converted into zeaxanthin by the enzyme encoded by the DNA sequence ⑥ (see Fig. 8).

Zeaxanthin is a xanthophyll whose color is in the spectrum ranging from yellow to orange, and it is an yellow pigment which is present in the seed of maize and has high safety for food. Zeaxanthin is contained in feeds for hen or colored carp and is an important pigment source for coloring them. In this connection, the zeaxanthin which was synthesized by the DNA sequences according to the present invention in the experimental example described below had the same stereochemistry as zeaxanthin described above.

One of the typical existing forms of the gene group and the individual DNA sequences is the same as defined in (1).

#### 15 (4) Gene group used for the synthesis of zeaxanthin-diglucoside

The gene group used for the synthesis of zeaxanthin-diglucoside which is one of yellow-orange carotenoids is a DNA sequence comprising the aforementioned DNA sequences ① - ⑥. In other words, the gene group used for the synthesis of zeaxanthin-diglucoside is formed by adding the DNA sequence ② to a DNA sequence used for the synthesis of zeaxanthin comprising the DNA sequences ①, ③, ④, ⑤ and ⑥. The gene group includes the one in which the respective DNA sequences constructing the gene group are present on one DNA strand or on different DNA strands individually, or the one which is constructed by the combination of the aforementioned ones according to necessities.

In the case that a plurality of DNA sequences are present on one DNA strand, the arrangement order and direction of the aforementioned DNA sequences ① - ⑥ may be optional provided that the genetic information is capable of expression, that is to say respective genes in a host are in a state of being transcribed and translated appropriately.

One of the typical existing forms of the gene group and the individual DNA sequences is the same as defined in (1).

The biosynthetic pathway of zeaxanthin-diglucoside in *E. coli* is explained as follows: geranylgeranyl pyrophosphate which is a substrate originally present in *E. coli* is converted into prephytoene pyrophosphate by the enzyme encoded by the DNA sequence ⑤, the prephytoene pyrophosphate is converted into phytoene by the enzyme encoded by the DNA sequence ①, the phytoene is then converted into lycopene by the enzyme encoded by the DNA sequence ④, and the lycopene is further converted into  $\beta$ -carotene by the enzyme encoded by the DNA sequence ③, the  $\beta$ -carotene is then converted into zeaxanthin by the enzyme encoded by the DNA sequence ⑤, and the zeaxanthin is finally converted into zeaxanthin-diglucoside by the enzyme encoded by the DNA sequence ② (see Fig. 8).

Zeaxanthin-diglucoside is a carotenoid glycoside having a high water solubility and a pigment which is soluble sufficiently in water at room temperature and exhibits clear yellow. Carotenoid pigments are generally hydrophobic and thus limited on their use as natural coloring materials in foods or the like. Therefore, zeaxanthin-diglucoside settles this defect. Zeaxanthin-diglucoside is isolated from edible plant saffron, *Crocus sativus* (Pure & Appl. Chem., 47, 121-128 (1976)), so that it is thought that its safety for food has been confirmed. Therefore, zeaxanthin-diglucoside is desirable as a yellow natural coloring material of foods or the like. In this connection, there has been heretofore no reports with reference to the isolation of zeaxanthin-diglucoside from microorganisms.

If carotenoid pigments such as lycopene,  $\beta$ -carotene, zeaxanthin and zeaxanthin-diglucoside are intended to be produced, the aforementioned DNA sequences ①, ④ and ⑤, the DNA sequences ①, ③, ④ and ⑤, the DNA sequences ①, ③, ④, ⑤ and ⑥, and the DNA sequences ① - ⑥ are required, respectively, on using *E. coli* as the host. However, when a host other than *E. coli*, particularly the one which is capable of producing carotenoids is used, it has a high possibility of containing also carotenoid precursors at further downstream in the biosynthesis, so that all of the aforementioned DNA sequences ①, ④ and ⑤ (for the production of lycopene), all of the DNA sequences ①, ③, ④ and ⑤ (for the production of  $\beta$ -carotene), all of the DNA sequences ①, ③, ④, ⑤ and ⑥ (for the production of zeaxanthin), or all of the DNA sequences ① - ⑥ (for the production of zeaxanthin-diglucoside) are not always required.

That is to say, only the DNA sequence(s) participating in the formation of an aimed carotenoid pigment from a carotenoid precursor present at the furthest downstream in the host may also be used in this case. Thus, when lycopene is intended to be produced as an aimed carotenoid in a host in which phytoene is preliminarily present, it is also possible to use only the DNA sequence ④ among the DNA sequences ①.

④ and ⑤.

It is also possible to make a host to produce, as the aimed carotenoid pigment relating compound, prephytoene pyrophosphate from geranylgeranyl pyrophosphate by using only the DNA sequence ⑤ of the present invention, or phytoene by using the DNA sequences ① and ⑤ of the present invention or, if the host contains prephytoene pyrophosphate, by using only the DNA sequence ①.

#### Acquirement of DNA sequences

A method for acquiring the DNA sequences ① - ⑥ which contain the nucleotide sequences coding for the amino acid sequences of the respective enzymes is the chemical synthesis of at least a part of their strand by the method of polynucleotide synthesis. However, if it is taken into consideration that a number of amino acids are bonded, it would be more preferable than the chemical synthesis to acquire the DNA sequences from the DNA library of Erwinia uredovora 20D3 ATTC 19321 according to a conventional method in the field of genetic engineering, for example, the hybridization method with a suitable probe.

The individual DNA sequences or the DNA sequence comprising all of these sequences are thus obtained.

#### Transformant

The aforementioned gene group comprising a plurality of the DNA sequences ① - ⑥ can be constituted by using the DNA sequences obtained as described above. The DNA sequence thus obtained contains genetic informations for making an enzyme participating in the formation of carotenoids, so that it can be introduced into an appropriate host by the biotechnological method to form a transformant and to produce an enzyme and in its turn a carotenoid pigment or a carotenoid pigment relating compound.

##### (1) Host

Plants and a variety of microorganisms, as far as a suitable host-vector system is present, can be the target of transformation by a vector comprising the aforementioned DNA sequences. However, the host is required to contain geranylgeranyl pyrophosphate which is a substrate compound of an enzyme for starting the carotenoid synthesis with use of the DNA sequences of the present invention, or a compound further downstream from it.

It is known that geranylgeranyl pyrophosphate is synthesized by dimethylallyltransferase which is a common enzyme at the initial stage of the biosynthesis of not only carotenoids but also sterols or terpenes [J. Biochem., 72, 1101-1108 (1972)]. Accordingly, if a cell which cannot synthesize carotenoids can synthesize sterols or terpenes, it probably contains geranylgeranyl pyrophosphate. It is believed that a cell contains at least one of sterols or terpenes.

Therefore, it is believed theoretically that almost all hosts are capable of synthesizing carotenoids by using the DNA sequences of the present invention as far as a suitable host-vector system is present.

As the hosts in which the host-vector system is present, there are mentioned plants such as Nicotiana tabacum, Petunia hybrida and the like, microorganism such as bacteria, for example Escherichia coli, Zymomonas mobilis and the like, and yeasts, for example Saccharomyces cerevisiae and the like.

##### (2) Transformation

It is confirmed for the first time by the present invention that the genetic informations present on the DNA sequences of the present invention has been expressed in microorganisms. However, the procedures or the methods for making the transformants (and the production of enzymes or in its turn carotenoid pigments or carotenoid pigment relating compounds by the transformants) are per se conventional in the fields of molecular biology, cell biology or genetic manipulation, and thus the procedures other than described below may be performed in accordance with these conventional techniques.

In order to express the gene of the DNA sequences according to the present invention in a host, it is necessary to insert the gene into a vector for introducing it into the host. As the vector used in this stage, there is used all of various known vectors such as pB1121 or the like for plants (Nicotiana tabacum, Petunia

hybrida); pUC19, pACYC184 or the like for *E. coli*; PZA22 or the like for *Zymomonas mobilis* (see Japanese Patent Laid-Open Publication No. 228278/87); and YEp13 or the like for yeast.

On the other hand, it is necessary to transcribe the DNA sequence of the present invention onto mRNA in order to express the gene of the DNA sequence in the host. For this purpose, a promoter as a signal for the transcription may be integrated into the 5'-upstream region from the DNA sequence of the present invention. A variety of promoters such as CaMV35S, NOS, TR1', TR2' (for plants); lac, Tc', CAT, trp (for *E. coli*); Tc', CAT (for *Zymomonas mobilis*); ADH1, GAL7, PGK, TRP1 (for yeast) and the like are known as for the promoters, and either of these promoters can be used in the present invention.

In the case of procaryote, it is necessary to place ribosome-binding site (SD sequence in *E. coli*) several base-upstream from the initiation codon (ATG).

In this connection, while the aforementioned manipulation is necessary for producing the enzyme protein, one or more of amino acids may be inserted into or added to the polypeptide which is illustrated in the specific ranges of Figs. 1 - 6 (e.g. the polypeptide A-B illustrated in Fig. 1), one or more of amino acids may be deleted, or replaced, as described above.

The transformation of the host with the plasmid thus obtained can be conducted optionally by an appropriate method which is conventionally used in the fields of genetic manipulation or cell biology. As for the general matters, there can be referred to appropriate publications or reviews; for example as for the transformation of microorganisms, T. Maniatis, E. F. Fritsch and J. Sambrook: "Molecular Cloning A Laboratory Manual", Cold Spring Harbor Laboratory (1982).

The transformant is the same as the host used, in its genotype, phenotype or bacteriological properties but for the new trait derived from the genetic information introduced by the DNA sequence of the present invention (that is, the production of an enzyme participating in the carotenoid formation and the synthesis of carotenoids or the like by the enzyme), the trait derived from the vector used and the deletion of the trait corresponding to the deletion of a part of the genetic information of the vector which might be caused on the recombination of genes. *Escherichia coli* JM109 (pCAR1) which is an example of the transformant according to the present invention is deposited as FERM BP-2377.

#### Expression of genetic information/production of carotenoids

The clone of the transformant obtained as described above produces mainly in the transformant an enzyme participating in the carotenoid formation, and a variety of carotenoids or carotenoid pigment relating compounds are synthesized by the enzyme.

Culture or the culturing condition of the transformant is essentially the same as those for the host used.

Carotenoids can be recovered by the methods, for example, illustrated in Experimental Examples 3 and 4 below.

Furthermore, each enzyme protein coded by each DNA sequence of the present invention is produced mainly in the cell in the case of the transformation of *E. coli*, and it can be recovered by an appropriate method.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 - 6 illustrate the nucleotide sequences in the DNA sequences ① - ⑥ in coding regions, and the amino acid sequences of proteins to be encoded, respectively.

Fig. 7 illustrates the KpnI-HindIII fragment which was acquired from *Erwinia uredovora* 20D3 ATCC 19321 and relates to the biosynthesis of carotenoids, that is the complete nucleotide sequence of the 6918 bp DNA sequence containing the DNA sequences in Figs. 1 - 6, and

Fig. 8 illustrates the function of the polypeptides encoded by the aforementioned DNA sequences ① - ⑥.

#### Experiments

All of strains used in the following experiments are deposited in ATCC or other deposition organizations and are freely available.

Experimental Example 1: Cloning of a gene cluster participating in the biosynthesis of a yellow pigment (referred to hereinafter as yellow pigment-synthesizing gene cluster)

5 (1) Preparation of total DNA

Total DNA was prepared from the cells of Erwinia uredovora 20D3 ATCC 19321 which had been proliferated until the early-stationary phase in 100 ml of LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl). Penicillin G (manufactured by Meiji Seika) was added to the culture medium so that it has a  
 10 concentration of 50 units/ml in the medium before 1 hour of the harvest of the cells. After harvesting the cells by centrifugation, this was washed with the TES buffer (20 mM tris, 10 mM EDTA, 0.1 M NaCl, pH 8), heat treated at 68 °C for 15 minutes and suspended in Solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8) containing 5 mg/ml of lysozyme (manufactured by Seikagaku Kogyo) and 100 µg/ml of RNase A (manufactured by Sigma). The suspension was incubated at 37 °C for a period of 30 minutes - 1 hour,  
 15 and pronase E (manufactured by Kaken Seiyaku) was added so that it had a concentration of 250 µg/ml before incubation at 37 °C for 10 minutes. Sodium N-lauroylsarcosine (manufactured by Nacalai tesque) was added so as it had the final concentration of 1%, and the mixture was agitated before incubation at 37 °C for several hours. Extraction was conducted several times with phenol/chloroform. While ethanol in volume of 2 equivalents was slowly added, the resulting total DNA was wound around a glass stick, rinsed  
 20 with 70% ethanol and dissolved in 2 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) to give the total DNA preparation.

25 (2) Construction of an Escherichia coli cosmid library and acquirement of E. coli transformants producing yellow pigments

Incubation was conducted with 1 unit of restriction enzyme Sau3AI per 50 µl of the total DNA preparation at 37 °C for 30 minutes before the inactivation treatment of the restriction enzyme at 68 °C for 10 minutes. Many fragments partially digested with Sau3AI were obtained in the neighbourhood of 40 kb  
 30 under this condition. After the ethanol precipitation of this reaction solution, this half portion was mixed with 2.5 µg of cosmid pJB8 which had been digested with BamHI and treated with alkaline phosphatase and 0.2 µg of a pJB8 Sall-BamHI right arm fragment (smaller fragment) which had been recovered from a gel, and 40 µl of the total amount was subjected to ligation reaction with T4 DNA ligase at 12 °C for 2 days. In this connection, the cosmid pJB8 had been previously purchased from Amersham. Restriction enzymes and  
 35 enzymes used for genetic manipulation were purchased from Boehringer-Mannheim, Takara Shuzo or Wako Pure Chemical Industries. This DNA in which the ligation reaction had been thus performed was used for in vitro packaging with a Gigapack Gold (manufactured by Stratagene, marketed from Funakoshi) to give a large amount of phage particles sufficient for construction of a cosmid library. The phage particles were infected with Escherichia coli DH1 (ATCC 33849). After the cells of E. coli DH1 infected were diluted so as  
 40 to be 100 colonies per plate, they were plated on a LB plate, cultured at 37 °C overnight and further at 30 °C for 6 hours or more. As a result, E. coli transformants producing yellow pigments appeared in a proportion of one colony per about 1,100 colonies. These E. coli transformants producing yellow pigments contained plasmids in which 33 - 47 kb Sau3AI partial digestion fragments were inserted into the pJB8.

45 (3) Location of a yellow pigment-synthesizing gene cluster

A yellow pigment-synthesizing gene cluster was inserted into the pJB8 as the 33 - 47 kb Sau3AI partial digestion fragments. One of these fragments was further subjected to partial digestion with Sau3AI, ligated  
 50 to the BamHI site of the E. coli vector pUC19 (purchased from Takara Shuzo), and used to transform Escherichia coli JM109 (manufactured by Takara Shuzo). To locate the yellow pigment-synthesizing gene cluster, plasmid DNA's were prepared from 50 E. coli transformants producing yellow pigments which appeared in the LB plate containing ampicillin, and analyzed by agarose gel electrophoresis. As a result, it was found that the smallest inserted fragment was of 8.2 kb. The plasmid containing this 8.2 kb fragment  
 55 was named as pCAR1 and E. coli JM109 harboring this plasmid was named as Escherichia coli JM109 (pCAR1). This strain produced the same yellow pigments as those of E. uredovora. The 8.2 kb fragment contained a KpnI site in the neighbourhood of the terminal at the lac promoter side and a HindIII site in the neighbourhood at the opposite side. After the 8.2 kb fragment was subjected to double digestion with

KpnI/HindIII (HindIII was partially digested; the 8.2 kb fragment had two HindIII sites), the KpnI-HindIII fragment (6.9 kb) was recovered from a gel and ligated to the KpnI-HindIII site of pUC18 (this hybrid plasmid was named as pCAR15). Upon the transformation of *E. coli* JM109, the *E. coli* transformant exhibited yellow and produced the same yellow pigments as those of *E. uredoovora*. Accordingly, it was found out that the genes required for the yellow pigment production was located on the KpnI-HindIII fragment (6.9 kb). That is to say, the fragment carrying the yellow pigment-synthesizing genes was capable of being reduced to a 6.9 kb in size.

## Experimental Example 2: Analysis of the yellow pigment-synthesizing gene cluster

### (1) Determination of the nucleotide sequence of the yellow pigment-synthesizing gene cluster

The complete nucleotide sequence of the 6.9 kb KpnI-HindIII fragment was determined by the kilo-sequence method using Deletion kit for kilo-sequence (manufactured by Takara Shuzo) and the dideoxy method according to Proc. Natl. Acad. Sci. USA, 74 5463-5467 (1977). As a result, it was found that the KpnI-HindIII fragment containing the yellow pigment-synthesizing genes (DNA strand) was 6918 base pairs (bp) in length and its GC content was 54%. The complete nucleotide sequence was shown in Fig. 7 (a) - (g). The KpnI site is represented by the base number 1.

### (2) Elucidation of yellow pigment-synthesizing gene cluster

The HindIII side of the 6918 bp fragment (DNA strand) containing the yellow pigment-synthesizing genes (right terminal side in Fig. 7) was deleted with Deletion kit for kilo-sequence. A hybrid plasmid (designated pCAR25) was constructed by inserting a 1 - 6503 fragment, which was obtained by deletion from the HindIII site to nucleotide position 6504, into pUC19. *E. coli* JM109 harboring pCAR25 [referred to hereinafter as *E. coli* (pCAR25)] exhibited yellow and produced the same yellow pigments as those of *E. uredoovora*. Therefore, it was thought that the region from the base number 6504 to 6918 in Fig. 7 was not required for yellow pigment production. The nucleotide sequence in the region from the base number 1 to 6503 in the 6918 bp DNA sequence containing the yellow pigment-synthesizing genes was analyzed. As a result, it was found that there were six open reading frames (ORFs). That is to say, there were an ORF coding for a polypeptide with a molecular weight of 32,583 from the base number 225 to 1130 (referred to as ORF1, which corresponds to A - B in Figs. 1 and 7), an ORF coding for a polypeptide with a molecular weight of 47,241 from the base number 1143 - 2435 (referred to as ORF2, which corresponds to C - D in Figs. 2 and 7), an ORF coding for a polypeptide with a molecular weight of 43,047 from the base number 2422 to 3567 (referred to as ORF3, which corresponds to E - F in Figs. 3 and 7), an ORF coding for a polypeptide with a molecular weight of 55,007 from the base number 3582 to 5057 (referred to as ORF4, which corresponds to G - H in Figs. 4 and 7), an ORF coding for a polypeptide with a molecular weight of 33,050 from the base number 5096 to 5983 (referred to as ORF5, which corresponds to I - J in Figs. 5 and 7), and an ORF coding for a polypeptide with a molecular weight of 19,816 from the base number 6452 to 5928 (referred to as ORF6, which corresponds to K - L in Figs. 6 and 7. Only this ORF6 has the opposite orientation with the others). In this connection, each ORF contained at positions several base-upstream from its initiation codon the SD (Shine-Dalgarno) sequence which is homologous with the 3'-region of 16S ribosomal RNA of *E. coli*. Thus, it was thought that polypeptides were in fact synthesized in *E. coli* by these six ORFs. This was confirmed by the following in vitro transcription-translation experiment.

That is to say, the in vitro transcription-translation analysis was carried out with DNA in which the plasmid pCAR25 containing ORF1 - ORF6 had been digested with *ScaI* and with DNAs in which respective fragments containing respective ORFs (containing the SD sequence) of ORF1 - ORF6 had been digested with appropriate restriction enzymes, isolated, inserted into pUC19 or pUC18 so that it was subjected to transcriptional read-through from a *lac* promoter, and then digested with *ScaI*. In this experiment, a Prokaryotic DNA-directed translation kit manufactured by Amersham was used. As a result, it was confirmed that the bands of polypeptides corresponding to the aforementioned respective ORFs were detected as the transcription-translation products.

Moreover, all of six ORFs were necessary for production of the same yellow pigments as those of *E. uredoovora* as described below (Experimental Examples 3, 4 and 5). From these results, ORF1, ORF2, ORF3, ORF4, ORF5 and ORF6 were designated as *zexA*, *zexB*, *zexC*, *zexD*, *zexE* and *zexF* genes.

respectively.

The base numbers in Figs. 1 - 6 were represented on the basis of the KpnI site in Fig. 7 as the base number 1 and correspond to each other. The marks A - L in Figs. 1 - 6 correspond to the marks A - L in Fig. 7. The DNA sequence from K to L in Fig. 6 was that of the complementary strand of the DNA sequence from K to L in Fig. 7. That is to say, the DNA sequence illustrated in Fig. 6 has the opposite orientation in transcription with the DNA sequences in Figs. 1 - 5 in the original DNA sequence (Fig. 7).

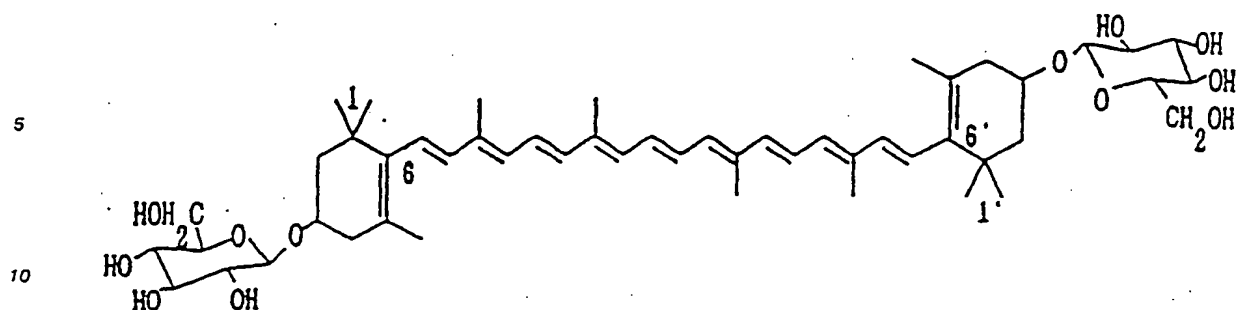
### (3) Analysis of homology by the DNA-DNA hybridization method

Total DNA of *Erwinia herbicola* Eho 10 ATCC 39368 was prepared in the same manner as in Experimental Example 1 (1). A 7.6 kb fragment containing the DNA sequence in Fig. 7 was cut out from the hybrid plasmid pCAR1 by KpnI digestion and labeled with DNA labeling & detection kit nonradioactive (manufactured by Boehringer-Mannheim) according to the DIG-ELISA method to give probe DNA. The homology of total DNAs (intact or KpnI digested) of *E. herbicola* Eho 10 ATCC 39368 and *E. uredo*vora 20D3 ATCC 19321 with this probe DNA was analyzed by the DNA-DNA hybridization method with the aforementioned DNA labeling & detection kit nonradioactive. As a result, the probe DNA was hybridized strongly with total DNA of the latter *E. uredo*vora 20D3 ATCC 19321, but not at all with total DNA of the former *E. herbicola* Eho 10 ATCC 39368. Also, the restriction map deduced from the DNA sequence in Fig. 7 was quite different from that reported in J. Bacteriol., 168, 607-612 (1986). It was concluded from the above described results that the DNA sequence in Fig. 7, that is, the DNA sequences useful for the synthesis of carotenoids according to the present invention exhibits no homology with the DNA sequence containing the yellow pigment-synthesizing genes of *E. herbicola* Eho 10 ATCC 39368.

### Experimental Example 3: Analysis of yellow pigments

*E. coli* (pCAR25) produced the same yellow pigments as those of *E. uredo*vora 20D3 ATCC 19321 and *E. herbicola* Eho 10 ATCC 39368, and its yield was 5 times higher than those of the former and 6 times higher than those of the latter (per dry weight). The cells harvested from 8 liters of 2 x YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) were extracted once with 1.2 liter of methanol. The methanol extract was evaporated to dryness, dissolved in methanol, and subjected to thin layer chromatography (TLC) with silica gel 60 (Merck) (developed with chloroform : methanol = 4:1). The yellow pigments were separated into 3 spots having R<sub>f</sub> values of 0.93, 0.62 and 0.30 by TLC. The yellow (to orange) pigment at the R<sub>f</sub> value of 0.30 which was the strongest spot was scraped up from the TLC plate, extracted with a small amount of methanol, loaded on a Sephadex LH-20 column for chromatography [30 cm x 3.0 cm (Ø)] and developed and eluted with methanol to give 4 mg of a pure product. The yellow (to orange) pigment obtained was sparingly soluble in organic solvents other than methanol and easily soluble in water, so that it was suggested that it might be a carotenoid glycoside. Such suggestion was also supported from a molecular weight of 892 by FD-MS spectrum (the mass of this pigment was larger than that of zeaxanthin (described hereinafter) by the mass of two glucose). When this substance was hydrolyzed with 1N HCl at 100 °C for 10 minutes, zeaxanthin was obtained. Then, acetylation was conducted according to the usual method. That is, the substance was dissolved in 10 ml of pyridine, large excess of acetic anhydride was added, and the mixture was stirred at room temperature and left standing overnight. After the completion of reaction, water was added to the mixture and chloroform extraction was carried out. The chloroform extract was concentrated and loaded on a silica gel column [30 cm x 3.0 cm (Ø)] for chromatography to develop and elute with chloroform. Measurement of <sup>1</sup>H-NMR gave the spectrum identical with the tetraacetyl derivative of zeaxanthin-β-diglucoside [Helvetica Chimica Acta, 57, 1641-1651 (1974)], so that the substance was identified as zeaxanthin-β-diglucoside (its structure being illustrated below).

The yield was 1.1 mg/g dry weight. The substance had a solubility of at least 2 mg in 100 ml of water and methanol, and water was superior to methanol in solubility of the substance. The substance had low solubilities in chloroform and acetone, and its solubilities were 0.5 mg in 100 ml of these solvents.



#### Experimental Example 4: Analysis of the metabolic intermediates of carotenoids

##### (1) Construction of various deletion plasmids

A hybrid plasmid (designated as pCAR16) was constructed by inserting a 1-6009 fragment, which was obtained by deletion to nucleotide position 6010 from the HindIII site (right terminal in Fig. 7) of the 6918 bp fragment containing the yellow pigment-synthesizing genes (DNA strand) (Fig. 7) using Deletion kit for kilo-sequence. pCAR16 contains the genes from zexA to zexE. Various deletion plasmids were constructed, as shown in Table 1, on the basis of the pCAR16 and the aforementioned hybrid plasmid pCAR25 (containing genes from zexA to zexF).

Table 1: Construction of various deletion plasmids

The number within parentheses behind the name of respective restriction enzymes represents the number of base at the initial recognition site of the restriction enzyme. The base numbers correspond to those in Figs. 1 - 6 and Fig. 7. Analysis of the metabolic intermediates of carotenoids was performed using the transformants of E. coli JM109 by various deletion plasmids [referred to hereinafter as E. coli (name of plasmid)].



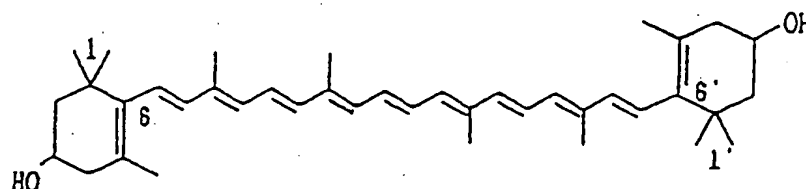
Table 1

Plasmid	Construction method	Genes functioning
pCAR25	See text	<u>zexA</u> <u>zexB</u> <u>zexC</u> <u>zexD</u> <u>zexE</u> <u>zexF</u>
pCAR25delB	Frame shift in <u>BstEII</u> (1235) of pCAR25	<u>zexA</u> <u>zexC</u> <u>zexD</u> <u>zexE</u> <u>zexF</u>
pCAR16	See text	<u>zexA</u> <u>zexB</u> <u>zexC</u> <u>zexD</u> <u>zexE</u>
pCAR16delB	Frame shift in <u>BstEII</u> (1235) of pCAR16	<u>zexA</u> <u>zexC</u> <u>zexD</u> <u>zexE</u>
pCAR16delC	Frame shift in <u>SnaBI</u> (3497) of pCAR16	<u>zexA</u> <u>zexB</u> <u>zexD</u> <u>zexE</u>
pCAR-ADE	Deletion of the <u>BstEII</u> (1235) - <u>SnaBI</u> (3497) fragment from pCAR16	<u>zexA</u> <u>zexD</u> <u>zexE</u>
pCAR-ADEF	Deletion of the <u>BstEII</u> (1235) - <u>SnaBI</u> (3497) fragment from pCAR25	<u>zexA</u> <u>zexD</u> <u>zexE</u> <u>zexF</u>
pCAR25delD	Frame shift in <u>BamHI</u> (3652) of pCAR25	<u>zexA</u> <u>zexB</u> <u>zexC</u> <u>zexE</u> <u>zexF</u>
pCAR-AE	Deletion of the <u>BstEII</u> (1235) - <u>BamHI</u> (3652) fragment from pCAR16	<u>zexA</u> <u>zexE</u>
pCAR-A	Insertion of the <u>KpnI</u> (1) - <u>BstEII</u> (1235) fragment in pUC19	<u>zexA</u>
pCAR-E	Insertion of the <u>Eco52I</u> (4926) - 6009 fragment in pUC19	<u>zexE</u>
pCAR25delE	Frame shift in <u>MluI</u> (5379) of pCAR25	<u>zexA</u> <u>zexB</u> <u>zexC</u> <u>zexD</u> <u>zexF</u>
pCAR25delA	Frame shift in <u>AvaI</u> (995) of pCAR25	<u>zexB</u> <u>zexC</u> <u>zexD</u> <u>zexE</u> <u>zexF</u>
pCAR-CDE	Insertion of the <u>Sall</u> (2295) - 6009 fragment in pUC19	<u>zexC</u> <u>zexD</u> <u>zexE</u>

## (2) Identification of zeaxanthin

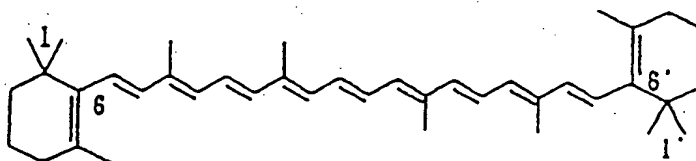
The cells harvested from 3 liters of 2 × YT medium of *E. coli* (pCAR25delB) (exhibiting orange) were extracted twice with 400 ml portions of acetone at low temperature, concentrated, then extracted with chloroform:methanol (9:1) and evaporated to dryness. This was subjected to silica gel column chromatography [30 cm. × 3.0 cm (Ø)]. After the column was washed with chloroform, an orange band was eluted with chloroform:methanol (100:1). This pigment was dissolved in ethanol, recrystallized at low temperature to give 8 mg of a pure product. The analysis by its UV-visible absorption, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR and FD-MS (m/e 568) spectra revealed that this substance had the same structure except for stereochemistry as zeaxanthin (β,β-carotene-3,3'-diol). It was then dissolved in diethyl ether : isopentane ethanol (5:5:2), and the CD spectrum was measured. As a result, it was found that this substance had a 3R,3'R-stereochemistry [Phytochemistry, 27, 3605-3609 (1988)]. Therefore, it was identified as zeaxanthin (β,β-carotene-3R,3'R-

diol), of which the structure is illustrated below. The yield was 2.2 mg/g dry weight. This substance corresponded to the yellow pigment having an R<sub>f</sub> value of 0.93 in Experimental Example (1).



### (3) Identification of $\beta$ -carotene

The cells harvested from 3 liters of LB medium of *E. coli* (pCAR16) (exhibiting orange) were extracted 3 times with 500 ml portions of cold methanol at low temperature and the methanol extract was further extracted with 1.5 liter of hexane. The hexane layer was concentrated and subjected to silica gel column chromatography [30 cm  $\times$  3.0 cm ( $\phi$ )]. Development and elution were conducted with hexane:ethyl acetate (50:1) to collect an orange band. The orange fraction was concentrated and recrystallized from ethanol to give 8 mg (reduced weight without moisture). This substance was presumed to belong to  $\beta$ -carotene from its UV-visible absorption spectrum, and a molecular weight of 536 by FD-MS spectrum also supported this presumption. Upon comparing this substance with the authentic sample (Sigma) of  $\beta$ -carotene by  $^{13}\text{C}$ -NMR spectrum, all of chemical shifts of carbons were identical with each other. Thus, this substance was identified as  $\beta$ -carotene (all-trans- $\beta$ , $\beta$ -carotene, of which the structure was illustrated below). It was also confirmed by the similar method that *E. coli* (pCAR16delB) accumulated the same  $\beta$ -carotene as described above. Its yield was 2.0 mg/g dry weight, which corresponded to 2-8 times (per dry weight) of the total carotenoid yield in carrot (Kintokininjin) culture cells described in Soshikibaiyou (The Tissue Culture), 13, 379-382 (1987).



### (4) Identification of lycopene

The cells harvested from 3 liters of LB medium of *E. coli* (pCAR16delC) (exhibiting red) were extracted once with 500 ml of cold methanol at low temperature, and the precipitate by centrifugation was extracted again with 1.5 liter of chloroform. The chloroform layer was concentrated and subjected to silica gel chromatography [30 cm  $\times$  3.0 cm ( $\phi$ )]. Development and elution were conducted with hexane:chloroform (1:1) to collect a red band. This fraction was concentrated. This substance was presumed to belong to lycopene from its UV-visible absorption spectrum, and a molecular weight of 536 by FD-MS spectrum also supported this presumption. Upon comparing this substance with the authentic sample (Sigma) of lycopene by  $^1\text{H}$ -NMR spectrum, all of chemical shifts of hydrogens were identical with each other. When, this substance and the authentic sample were subjected to TLC with silica gel 60 (Merck) [developed with hexane:chloroform (50:1)] and with RP-18 [developed with methanol:chloroform (4:1)], the displacement distances of these samples were completely equal to each other. Thus this substance was identified as lycopene (all-trans- $\beta$ , $\beta$ -carotene, of which the structure was illustrated below). It was also confirmed by the similar method that *E. coli* (pCAR-ADE) and *E. coli* (pCAR-ADEF) accumulated the same lycopene as described above. The yield of the former was 2.0 mg/g dry weight, which corresponded to 2 times (per dry weight) of the total carotenoid yield in a hyperproduction derivative of carrot (Kintokininjin) culture cells described in Soshikibaiyou (The Tissue Culture), 13, 379-382 (1987).



#### (5) Identification of phytoene

The cells harvested from 1.5 liter of 2 x YT medium of *E. coli* (pCAR-AE) were extracted twice with 200 ml portions of acetone and twice with 100 ml portions of hexane, and evaporated to dryness. This was subjected to silica gel chromatography [30 cm x 3.0 cm (Ø)]. Development and elution were conducted with hexane:chloroform (1:1) to collect a band which had a strong UV absorption, and it was confirmed to be phytoene by its UV absorption spectrum. It was further subjected to LH-20 column chromatography [30 cm x 3.0 cm (Ø)]. Development and elution were conducted with chloroform:methanol (1:1) to give 4 mg of a pure product. The comparison of the <sup>1</sup>H-NMR spectrum of this substance with the <sup>1</sup>H-NMR spectra of *trans*- and *cis*-phytoen (J. Magnetic Resonance, 10, 43-50 (1973)) showed this substance to be a mixture of the *trans*- and *cis*-isomers. Isomerization from *cis*-isomer to *trans*-isomer hardly occurs, and thus it was judged that such a mixture was produced as a result of *trans*-*cis* isomerization in the course of the purification. Therefore, it was concluded that the original phytoene was the *trans*-type phytoene (all-*trans*-phytoene, of which the structure was shown below). It was also confirmed by the similar method that *E. coli* (pCAR25delD) accumulated the same phytoene as described above.



#### Experimental Example 5: Identification of carotenoid biosynthesis genes

From the facts that *E. coli* (pCAR25) produced zeaxanthin-diglucoside and that *E. coli* (pCAR25delB) harboring a plasmid, in which *zexB* had been removed from pCAR25, accumulated zeaxanthin, it was found that the *zexB* gene encoded the glycosylation enzyme which was capable of converting zeaxanthin into zeaxanthin-diglucoside. Similarly, from the fact that *E. coli* (pCAR16delB) harboring a plasmid, in which *zexF* had been removed from pCAR25delB, accumulated  $\beta$ -carotene, it was found that the *zexF* gene encoded the hydroxylation enzyme which was capable of converting  $\beta$ -carotene into zeaxanthin. Similarly, from the fact that the *E. coli* (pCAR-ADE) harboring a plasmid, in which *zexC* had been removed from pCAR16delB, accumulated lycopene, it was found that the *zexC* gene encoded the cyclization enzyme which was capable of converting lycopene into  $\beta$ -carotene. Also, *E. coli* (pCAR-ADEF) carrying both of the *zexA*, *zexD* and *zexE* genes required for producing lycopene and the *zexF* gene encoding the hydroxylation enzyme was able to synthesize only lycopene. This demonstrates directly that the hydroxylation reaction in carotenoid biosynthesis occurs after the cyclization reaction. Further, from the facts that *E. coli* (pCAR-ADE) accumulated lycopene and that *E. coli* (pCAR-AE) harboring a plasmid, in which the *zexD* gene had been removed from pCAR-ADE, accumulated phytoene, it was found that the *zexD* gene encoded the desaturation enzyme which was capable of converting phytoene into lycopene. *E. coli* (pCAR-A) and *E. coli* (pCAR-E) were not able to produce phytoene. It was thought from this result that both of the *zexA* and *zexE* genes were required for producing phytoene in *E. coli*. *zexE* and *zexA* were identified as the gene for the conversion of geranylgeranyl pyrophosphate into prephytoene pyrophosphate and that for the conversion of prephytoene pyrophosphate into phytoene, by comparing their putative amino acid sequence with those of *crtB* and *crtE* gene products in a photo synthetic bacterium *Rhodobacter capsulatus* [Mol. Gen. Genet., 216, 254-268 (1988)]. From these analyses described above, all of the six *zex* genes have been identified and the biosynthetic pathway of carotenoids have also been clear. These results are listed in Fig. 8.

*E. coli* (pCAR25delE) accumulated no detectable carotenoid intermediate, while *E. coli* (pCAR25delA) and *E. coli* (pCAR-CDE) were able to produce a small amount of carotenoids. That is to say, *E. coli* (pCAR25delA) and *E. coli* (pCAR-CDE) produced 4% of zeaxanthin-diglucoside and 2% of  $\beta$ -carotene as

compared with the *E. coli* (pCAR25) and the *E. coli* (pCAR16delb), respectively. This result suggests that the reaction from prephytoene pyrophosphate to phytoene may occur non-enzymatically notwithstanding the yield being trace.

As described above, the detailed biosynthetic pathway of carotenoids including general and famous carotenoids such as lycopene,  $\beta$ -carotene and zeaxanthin and water soluble carotenoid such as zeaxanthin-diglucoside were for the first time elucidated, and the gene cluster useful for these biosynthesis was capable of being acquired for the first time. In this connection, lycopene,  $\beta$ -carotene and zeaxanthin which were produced by the genes in the aforementioned Experimental Examples were stereochemically identical with those derived from higher plants [T.W. Goodwin: "Plant Pigments", Academic Press (1988)].

As for zeaxanthin-diglucoside, the isolation from a plant was only reported [Pure & Appl. Chem., 47, 121-128 (1976)], but its isolation from microorganisms has not been reported.

#### Experimental Example 6: Synthesis of carotenoids in *Zymomonas*

*Zymomonas mobilis* is a facultative anaerobic ethanol-producing bacterium. It has a higher ethanol producing rate than that of yeast (*Saccharomyces cerevisiae*), so that it is preferable as a fuel alcohol-producing bacterium in future. Also, *Zymomonas* has a special metabolic pathway, Entner-Doudoroff but not glycolytic pathway and cannot produce carotenoids. In order to add further values to this bacterium, the carotenoid biosynthesis genes were introduced into *Zymomonas*.

The 7.6 kb fragment containing the DNA sequence shown in Fig. 7 was cut out from the hybrid plasmid pCAR1 by KpnI digestion and treated with DNA polymerase I (Klenow enzyme). The fragment thus treated was ligated to the EcoRV site of the cloning vector pZA22 for *Zymomonas* [see Agric. Biol. Chem., 50, 3201-3203 (1986) and Japanese Patent Laid-Open Publication No. 228278/87] to construct a hybrid plasmid pZACAR1. Also, the 1-6009 fragment in the DNA sequence in Fig. 7 was cut out from pCAR16 by KpnI/EcoRI digestion and treated with DNA polymerase I (Klenow enzyme). The fragment thus treated was ligated to the EcoRV site of pZA22 to construct a hybrid plasmid pZACAR16. The orientation of the inserted fragments in these plasmids were opposite with the orientation of the Tc<sup>r</sup> gene on taking the orientation in Fig. 7 as the normal one. These plasmids were introduced into *Z. mobilis* NRRL B-14023 by conjugal transfer with the helper plasmid pRK2013 (ATCC 37159) and stably maintained in this strain. *Z. mobilis* NRRL B-14023 in which pZACAR1 and pZACAR16 had been introduced exhibited yellow, and produced zeaxanthin-diglucoside in an amount of 0.28 mg/g dry weight and  $\beta$ -carotene in an amount of 0.14 mg/g dry weight, respectively. Therefore, carotenoids were successfully produced in *Zymomonas* by the carotenoid biosynthesis genes according to the present invention.

#### Deposition of Microorganism

Microorganism relating to the present invention is deposited at Fermentation Research Institute, Japan as follows:

Microorganism	Accession number	Date of deposit
<i>Escherichia coli</i> JM109 (pCAR1)	FERM BP 2377	April 11, 1989

#### Claims

1. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting prephytoene pyrophosphate into phytoene and whose amino acid sequence corresponds substantially to the amino acid sequence from A to B shown in Figs. 1 (a) and (b).

2. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting zeaxanthin into zeaxanthin-diglucoside and whose amino acid sequence corresponds substantially to the amino acid sequence from C to D shown in Figs. 2 (a) and (b).

3. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting lycopene into  $\beta$ -carotene and whose amino acid sequence corresponds substantially to the amino acid sequence

from E to F shown in Figs. 3 (a) and (b).

4. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting phytoene into lycopene and whose amino acid sequence corresponds substantially to the amino acid sequence from G to H shown in Figs. 4 (a), (b) and (c).

5. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting geranyl-geranyl pyrophosphate into prephytoene pyrophosphate and whose amino acid sequence corresponds substantially to the amino acid sequence from I to J shown in Figs. 5 (a) and (b).

6. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting  $\beta$ -carotene into zeaxanthin and whose amino acid sequence corresponds substantially to the amino acid sequence from K to L shown in Fig. 6.

7. A process for producing a carotenoid compound which is selected from the group consisting of prephytoene pyrophosphate, phytoene, lycopene,  $\beta$ -carotene, zeaxanthin and zeaxanthin-diglucoside, which comprises transforming a host with at least one of the DNA sequences according to claims 1-6, and culturing the transformant.

230 240 250 260 270 280  
 ATGACGGTCTGCGCAAAAAACACGTTTCATCTCACTCGCGATGCTGCGGAGCAGTTACTG  
 MetThrValCysAlaLysLysHisValHisLeuThrArgAspAlaAlaGluGlnLeuLeu  
 A  
 290 300 310 320 330 340  
 GCTGATATTGATCGACGCCTTGATCAGTTATTGCCCGTGGAGGGAGAACGGGATGTTGTG  
 AlaAspIleAspArgArgLeuAspGlnLeuLeuProValGluGlyGluArgAspValVal  
 350 360 370 380 390 400  
 GGTGCCGCGATGCGTGAAGGTGCGCTGGCACCAGGAAACGTATTCGCCCCATGTTGCTG  
 GlyAlaAlaMetArgGluGlyAlaLeuAlaProGlyLysArgIleArgProMetLeuLeu  
 410 420 430 440 450 460  
 TTGCTGACCGCCCGCGATCTGGGTTGCGCTGTCAGCCATGACGGATTACTGGATTTGGCC  
 LeuLeuThrAlaArgAspLeuGlyCysAlaValSerHisAspGlyLeuLeuAspLeuAla  
 470 480 490 500 510 520  
 TGTGCGGTGGAAATGGTCCACGCGGCTTCGCTGATCCTTGACGATATGCCCTGCATGGAC  
 CysAlaValGluMetValHisAlaAlaSerLeuIleLeuAspAspMetProCysMetAsp  
 530 540 550 560 570 580  
 GATGCGAAGCTGCGGCGCGGACGCCCTACCATTTCATTCTCATTACGGAGAGCATGTGGCA  
 AspAlaLysLeuArgArgGlyArgProThrIleHisSerHisTyrGlyGluHisValAla  
 590 600 610 620 630 640  
 ATACTGGCGGCGGTTGCCTTGCTGAGTAAAGCCTTTGGCGTAATTGCCGATGCAGATGGC  
 IleLeuAlaAlaValAlaLeuLeuSerLysAlaPheGlyValIleAlaAspAlaAspGly  
 650 660 670 680 690 700  
 CTCACGCCGCTGGCAAAAATCGGGCGGTTTCTGAAGTGTCAAACGCCATCGGCATGCAA  
 LeuThrProLeuAlaLysAsnArgAlaValSerGluLeuSerAsnAlaIleGlyMetGln  
 710 720 730 740 750 760  
 GGATTGGTTCAGGGTCAGTTCAAGGATCTGTCTGAAGGGGATAAGCCGCGCAGCGCTGAA  
 GlyLeuValGlnGlyGlnPheLysAspLeuSerGluGlyAspLysProArgSerAlaGlu  
 770 780 790 800 810 820  
 GCTATTTTGTATGACGAATCACTTTAAACCAGCACGCTGTTTTGTGCCTCCATGCAGATG  
 AlaIleLeuMetThrAsnHisPheLysThrSerThrLeuPheCysAlaSerMetGlnMet  
 830 840 850 860 870 880  
 GCCTCGATTGTTGCGAATGCCTCCAGCGAAGCGCGTGATTGCCTGCATCGTTTTTCACTT  
 AlaSerIleValAlaAsnAlaSerSerGluAlaArgAspCysLeuHisArgPheSerLeu

FIG. 1 (a)

890 900 910 920 930 940  
GATCTTGGTCAGGCATTTCAACTGCTGGACGATTTGACCGATGGCATGACCGACACCGGT  
AspLeuGlyGlnAlaPheGlnLeuLeuAspAspLeuThrAspGlyMetThrAspThrGly

950 960 970 980 990 1000  
AAGGATAGCAATCAGGACGCCGGTAAATCGACGCTGGTCAATCTGTTAGGCCCGAGGGCG  
LysAspSerAsnGlnAspAlaGlyLysSerThrLeuValAsnLeuLeuGlyProArgAla

1010 1020 1030 1040 1050 1060  
GTTGAAGAACGTCTGAGACAACATCTTCAGCTTGCCAGTGAGCATCTCTCTGCGGCCTGC  
ValGluGluArgLeuArgGlnHisLeuGlnLeuAlaSerGluHisLeuSerAlaAlaCys

1070 1080 1090 1100 1110 1120  
CAACACGGGCACGCCACTCAACATTTTATTCAGGCCTGGTTTGACAAAAAAGCTCGCTGCC  
GlnHisGlyHisAlaThrGlnHisPheIleGlnAlaTrpPheAspLysLysLeuAlaAla

1130  
GTCAGTTAA  
ValSer\*\*\*  
↑  
B

FIG. 1 (b)

1150 1160 1170 1180 1190 1200  
 ATGAGCCATTTTCGCGGCGATCGCACCGCCTTTTACAGCCATGTTTCGCGCATTACAGAAT  
 MetSerHisPheAlaAlaIleAlaProProPheTyrSerHisValArgAlaLeuGlnAsn  
 ↑  
 C

1210 1220 1230 1240 1250 1260  
 CTCGCTCAGGAACTGGTCGCGCGCGGTTCATCGGGTGACCTTTATTTCAGCAATACGATATT  
 LeuAlaGlnGluLeuValAlaArgGlyHisArgValThrPheIleGlnGlnTyrAspIle

1270 1280 1290 1300 1310 1320  
 AAACACTTGATCGATAGCGAAACCATTGGATTTCATTCCGTCGGGACAGACAGCCATCCC  
 LysHisLeuIleAspSerGluThrIleGlyPheHisSerValGlyThrAspSerHisPro

1330 1340 1350 1360 1370 1380  
 CCCGGCGCGTTAACGCGCGTGCTACACCTGGCGGCTCATCCTCTGGGGCCGTCAATGCTG  
 ProGlyAlaLeuThrArgValLeuHisLeuAlaAlaHisProLeuGlyProSerMetLeu

1390 1400 1410 1420 1430 1440  
 AAGCTCATCAATGAAATGGCGCGCACCCACCGATATGCTGTGCCGCGAACTCCCCCAGGCA  
 LysLeuIleAsnGluMetAlaArgThrThrAspMetLeuCysArgGluLeuProGlnAla

1450 1460 1470 1480 1490 1500  
 TTTAACGATCTGGCCGTCGATGGCGTCATTGTTGATCAAATGGAACCGGCAGGCGCGCTC  
 PheAsnAspLeuAlaValAspGlyValIleValAspGlnMetGluProAlaGlyAlaLeu

1510 1520 1530 1540 1550 1560  
 GTTGCTGAAGCACTGGGACTGCCGTTTATCTCTGTGCGCTGCGCGCTGCCTCTCAATCGT  
 ValAlaGluAlaLeuGlyLeuProPheIleSerValAlaCysAlaLeuProLeuAsnArg

1570 1580 1590 1600 1610 1620  
 GAACCGGATATGCCCTGGCGGTTATGCCTTTTCGAATACGGGACCAGCGACGCGGCTCGC  
 GluProAspMetProLeuAlaValMetProPheGluTyrGlyThrSerAspAlaAlaArg

1630 1640 1650 1660 1670 1680  
 GAACGTTATGCCGCCAGTGAAAAATTTATGACTGGCTAATGCGTCGTCATGACCGTGTC  
 GluArgTyrAlaAlaSerGluLysIleTyrAspTrpLeuMetArgArgHisAspArgVal

1690 1700 1710 1720 1730 1740  
 ATTGCCGAACACAGCCACAGAATGGGCTTAGCCCCCGGCAAAAGCTTCACCACTGTTTT  
 IleAlaGluHisSerHisArgMetGlyLeuAlaProArgGlnLysLeuHisGlnCysPhe

1750 1760 1770 1780 1790 1800  
 TCGCCACTGGCGCAAATCAGCCAGCTTGTTTCCTGAACTGGATTTTCCCCGCAAAGCGTTA  
 SerProLeuAlaGlnIleSerGlnLeuValProGluLeuAspPheProArgLysAlaLeu

FIG. 2 (a)



1810 1820 1830 1840 1850 1860  
CCGGCTTGTTCATGCCGTGGGCTCTGCGCGAAACGCACGCCGTCAACGTCTTCA  
ProAlaCysPheHisAlaValGlyProLeuArgGluThrHisAlaProSerThrSerSer

1870 1880 1890 1900 1910 1920  
TCCCGTTATTTTACATCCTCAGAAAAACCCCGGATTTTCGCCTCGCTGGGCACGCTTCAG  
SerArgTyrPheThrSerSerGluLysProArgIlePheAlaSerLeuGlyThrLeuGln

1930 1940 1950 1960 1970 1980  
GGACACCGTTATGGGCTGTTTAAACGATAGTGAAAGCCTGTGAAGAAATTGACGGTCAG  
GlyHisArgTyrGlyLeuPheLysThrIleValLysAlaCysGluGluIleAspGlyGln

1990 2000 2010 2020 2030 2040  
CTCCTGTTAGCCCACTGTGGTCGTCTTACGGACTCTCAGTGTGAAGAGCTGGCGCGAAGC  
LeuLeuLeuAlaHisCysGlyArgLeuThrAspSerGlnCysGluGluLeuAlaArgSer

2050 2060 2070 2080 2090 2100  
CGTCATACACAGGTGGTGGATTTTGCCGATCAGTCAGCCGCGCTGTCTCAGGCGCAGCTG  
ArgHisThrGlnValValAspPheAlaAspGlnSerAlaAlaLeuSerGlnAlaGlnLeu

2110 2120 2130 2140 2150 2160  
GCGATCACCCACGGCGGCATGAATACGGTACTGGACGCGATTAATTACCGGACGCCCTT  
AlaIleThrHisGlyGlyMetAsnThrValLeuAspAlaIleAsnTyrArgThrProLeu

2170 2180 2190 2200 2210 2220  
TTAGCGCTTCCGCTGGCCTTTGATCAGCCCGGCGTCGCGTCACGCATCGTTTATCACGGC  
LeuAlaLeuProLeuAlaPheAspGlnProGlyValAlaSerArgIleValTyrHisGly

2230 2240 2250 2260 2270 2280  
ATCGGCAAGCGTGCTTCCCGCTTTACCACCAGCCATGCTTTGGCTCGTCAGATGCGTTCA  
IleGlyLysArgAlaSerArgPheThrThrSerHisAlaLeuAlaArgGlnMetArgSer

2290 2300 2310 2320 2330 2340  
TTGCTGACCAACGTCGACTTTCAGCAGCGCATGGCGAAAATCCAGACAGCCCTTCGTTTG  
LeuLeuThrAsnValAspPheGlnGlnArgMetAlaLysIleGlnThrAlaLeuArgLeu

2350 2360 2370 2380 2390 2400  
GCAGGGGGCACCATGGCCGCTGCCGATATCATTGAGCAGGTTATGTGCACCGGTCAGCCT  
AlaGlyGlyThrMetAlaAlaAlaAspIleIleGluGlnValMetCysThrGlyGlnPro

D  
↓

2410 2420 2430  
GTCTTAAGTGGGAGCGGCTATGCAACCGCATTATGA  
ValLeuSerGlySerGlyTyrAlaThrAlaLeu\*\*\*

FIG. 2 (b)

2430 2440 2450 2460 2470 2480  
ATGCAACCGCATTATGATCTGATTCTCGTGGGGGCTGGACTCGCGAATGGCCTTATCGCC  
MetGlnProHisTyrAspLeuIleLeuValGlyAlaGlyLeuAlaAsnGlyLeuIleAla  
↑  
E 2490 2500 2510 2520 2530 2540  
CTGCGTCTTCAGCAGCAGCAACCTGATATGCGTATTTTGCTTATCGACGCCGCACCCAG  
LeuArgLeuGlnGlnGlnGlnProAspMetArgIleLeuLeuIleAspAlaAlaProGln  
2550 2560 2570 2580 2590 2600  
GCGGGCGGGAATCATACGTGGTCATTTACCACGATGATTTGACTGAGAGCCAACATCGT  
AlaGlyGlyAsnHisThrTrpSerPheHisHisAspAspLeuThrGluSerGlnHisArg  
2610 2620 2630 2640 2650 2660  
TGGATAGCTCCGCTGGTGGTTCATCACTGGCCCGACTATCAGGTACGCTTTCCACACGC  
TrpIleAlaProLeuValValHisHisTrpProAspTyrGlnValArgPheProThrArg  
2670 2680 2690 2700 2710 2720  
CGTCGTAAGCTGAACAGCGGCTACTTTTGTATTACTTCTCAGCGTTTCGCTGAGGTTTTA  
ArgArgLysLeuAsnSerGlyTyrPheCysIleThrSerGlnArgPheAlaGluValLeu  
2730 2740 2750 2760 2770 2780  
CAGCGACAGTTTGGCCCGCACTTGTGGATGGATACCGCGGTTCGAGAGGTTAATGCGGAA  
GlnArgGlnPheGlyProHisLeuTrpMetAspThrAlaValAlaGluValAsnAlaGlu  
2790 2800 2810 2820 2830 2840  
TCTGTTTCGGTTGAAAAAGGGTCAGGTTATCGGTGCCCGCGCGGTGATTGACGGGCGGGGT  
SerValArgLeuLysLysGlyGlnValIleGlyAlaArgAlaValIleAspGlyArgGly  
2850 2860 2870 2880 2890 2900  
TATGCGGCAAATTCAGCACTGAGCGTGGGCTTCCAGGCGTTTATTGGCCAGGAATGGCGA  
TyrAlaAlaAsnSerAlaLeuSerValGlyPheGlnAlaPheIleGlyGlnGluTrpArg  
2910 2920 2930 2940 2950 2960  
TTGAGCCACCCGCATGGTTTATCGTCTCCATTATCATGGATGCCACGGTCGATCAGCAA  
LeuSerHisProHisGlyLeuSerSerProIleIleMetAspAlaThrValAspGlnGln  
2970 2980 2990 3000 3010 3020  
AATGGTTATCGCTTCGTGTACAGCCTGCCGCTCTCGCCGACCAGATTGTTAATTGAAGAC  
AsnGlyTyrArgPheValTyrSerLeuProLeuSerProThrArgLeuLeuIleGluAsp  
3030 3040 3050 3060 3070 3080  
ACGCACTATATTGATAATGCGACATTAGATCCTGAATGCGCGCGGCAAAATATTTGCGAC  
ThrHisTyrIleAspAsnAlaThrLeuAspProGluCysAlaArgGlnAsnIleCysAsp

FIG. 3 (a)

3090 3100 3110 3120 3130 3140  
TATGCCGCGCAACAGGGTTGGCAGCTTCAGACACTGCTGCGAGAAGAACAGGGCGCCTTA  
TyrAlaAlaGlnGlnGlyTrpGlnLeuGlnThrLeuLeuArgGluGluGlnGlyAlaLeu

3150 3160 3170 3180 3190 3200  
CCCATTACTCTGTCTGGGCAATGCCGACGCATTCTGGCAGCAGCGCCCCCTGGCCTGTAGT  
ProIleThrLeuSerGlyAsnAlaAspAlaPheTrpGlnGlnArgProLeuAlaCysSer

3210 3220 3230 3240 3250 3260  
GGATTACGTGCCGGTCTGTTCCATCCTACCACGGCTATTCACTGCCGCTGGCGGTTGCC  
GlyLeuArgAlaGlyLeuPheHisProThrThrGlyTyrSerLeuProLeuAlaValAla

3270 3280 3290 3300 3310 3320  
GTGGCCGACCGCCTGAGTGCACCTTGATGTCTTTACGTCGGCCTCAATTCACCATGCCATT  
ValAlaAspArgLeuSerAlaLeuAspValPheThrSerAlaSerIleHisHisAlaIle

3330 3340 3350 3360 3370 3380  
ACGCATTTTGGCCGCGAGCGCTGGCAGCAGCAGGGCTTTTCCGCATGCTGAATCGCATG  
ThrHisPheAlaArgGluArgTrpGlnGlnGlnGlyPhePheArgMetLeuAsnArgMet

3390 3400 3410 3420 3430 3440  
CTGTTTTTAGCCGGACCCGCCGATTACGCTGGCGGGTTATGCAGCGTTTTTATGGTTTA  
LeuPheLeuAlaGlyProAlaAspSerArgTrpArgValMetGlnArgPheTyrGlyLeu

3450 3460 3470 3480 3490 3500  
CCTGAAGATTTAATTGCCCGTTTTTATGCGGGAAACTCACGCTGACCGATCGGCTACGT  
ProGluAspLeuIleAlaArgPheTyrAlaGlyLysLeuThrLeuThrAspArgLeuArg

3510 3520 3530 3540 3550 3560  
ATTCTGAGCGGCAAGCCGCTGTTCCGGTATTAGCAGCATTGCAAGCCATTATGACGACT  
IleLeuSerGlyLysProProValProValLeuAlaAlaLeuGlnAlaIleMetThrThr

3570  
CATCGTTAA  
HisArg\*\*\*  
↑  
F

FIG. 3 (b)

3590 3600 3610 3620 3630 3640  
 ATGAAACCAACTACGGTAATTGGTGCAGGCTTCGGTGGCCTGGCACTGGCAATTCGTCTA  
 MetLysProThrThrValIleGlyAlaGlyPheGlyGlyLeuAlaLeuAlaIleArgLeu  
 ↑  
 G 3650 3660 3670 3680 3690 3700  
 CAAGCTGCGGGGATCCCCGTCTTACTGCTTGAACAACGTGATAAACCCGGCGGTTCGGGCT  
 GlnAlaAlaGlyIleProValLeuLeuLeuGluGlnArgAspLysProGlyGlyArgAla  
  
 3710 3720 3730 3740 3750 3760  
 TATGTCTACGAGGATCAGGGGTTTACCTTTGATGCAGGCCCGACGGTTATCACCGATCCC  
 TyrValTyrGluAspGlnGlyPheThrPheAspAlaGlyProThrValIleThrAspPro  
  
 3770 3780 3790 3800 3810 3820  
 AGTGCCATTGAAGAACTGTTTGCCTGGCAGGAAAACAGTTAAAAGAGTATGTCGAACTG  
 SerAlaIleGluGluLeuPheAlaLeuAlaGlyLysGlnLeuLysGluTyrValGluLeu  
  
 3830 3840 3850 3860 3870 3880  
 CTGCCGGTTACGCCGTTTTACCGCCTGTGTTGGGAGTCAGGGAAGGTCTTTAATTACGAT  
 LeuProValThrProPheTyrArgLeuCysTrpGluSerGlyLysValPheAsnTyrAsp  
  
 3890 3900 3910 3920 3930 3940  
 AACGATCAAACCCGGCTCGAAGCGCAGATTCAGCAGTTTAATCCCCGCGATGTCTGAAGGT  
 AsnAspGlnThrArgLeuGluAlaGlnIleGlnGlnPheAsnProArgAspValGluGly  
  
 3950 3960 3970 3980 3990 4000  
 TATCGTCAGTTTCTGGACTATTCACGCGCGGTGTTTAAAGAAGGCTATCTAAAGCTCGGT  
 TyrArgGlnPheLeuAspTyrSerArgAlaValPheLysGluGlyTyrLeuLysLeuGly  
  
 4010 4020 4030 4040 4050 4060  
 ACTGTCCCTTTTTTATCGTTCAGAGACATGCTTCGCGCCGCACCTCAACTGGCGAACTG  
 ThrValProPheLeuSerPheArgAspMetLeuArgAlaAlaProGlnLeuAlaLysLeu  
  
 4070 4080 4090 4100 4110 4120  
 CAGGCATGGAGAAGCGTTTACAGTAAGGTTGCCAGTTACATCGAAGATGAACATCTGCGC  
 GlnAlaTrpArgSerValTyrSerLysValAlaSerTyrIleGluAspGluHisLeuArg  
  
 4130 4140 4150 4160 4170 4180  
 CAGGCGTTTTCTTTCCACTCGCTGTTGGTGGGCGGCAATCCCTTCGCCACCTCATCCATT  
 GlnAlaPheSerPheHisSerLeuLeuValGlyGlyAsnProPheAlaThrSerSerIle  
  
 4190 4200 4210 4220 4230 4240  
 TATACGTTGATACACGCGCTGGAGCGTGAGTGGGGCGTCTGGTTTCCGCGTGGCGGCACC  
 TyrThrLeuIleHisAlaLeuGluArgGluTrpGlyValTrpPheProArgGlyGlyThr

FIG. 4 (a)

4250 4260 4270 4280 4290 4300  
 GGCGCATTAGTTCAGGGGATGATAAAGCTGTTTCAGGATCTGGGTGGCGAAGTCGTGTTA  
 GlyAlaLeuValGlnGlyMetIleLysLeuPheGlnAspLeuGlyGlyGluValValLeu

4310 4320 4330 4340 4350 4360  
 AACGCCAGAGTCAGCCATATGGAAACGACAGGAAACAAGATTGAAGCCGTGCATTTAGAG  
 AsnAlaArgValSerHisMetGluThrThrGlyAsnLysIleGluAlaValHisLeuGlu

4370 4380 4390 4400 4410 4420  
 GACGGTCGCAGGTTCTGACGCAAGCCGTCGCGTCAAATGCAGATGTGGTTCATACCTAT  
 AspGlyArgArgPheLeuThrGlnAlaValAlaSerAsnAlaAspValValHisThrTyr

4430 4440 4450 4460 4470 4480  
 CGCGACCTGTTAAGCCAGCACCCCTGCCGCGGTTAAGCAGTCCAACAACTGCAGACTAAG  
 ArgAspLeuLeuSerGlnHisProAlaAlaValLysGlnSerAsnLysLeuGlnThrLys

4490 4500 4510 4520 4530 4540  
 CGCATGAGTAACTCTCTGTTTGTGCTCTATTTTGGTTTGAATCACCATCATGATCAGCTC  
 ArgMetSerAsnSerLeuPheValLeuTyrPheGlyLeuAsnHisHisHisAspGlnLeu

4550 4560 4570 4580 4590 4600  
 GCGCATCACACGGTTTTGTTTCGGCCCGCGTTACCGCGAGCTGATTGACGAAATTTTAAAT  
 AlaHisHisThrValCysPheGlyProArgTyrArgGluLeuIleAspGluIlePheAsn

4610 4620 4630 4640 4650 4660  
 CATGATGGCCTCGCAGAGGACTTCTCACTTTATCTGCACGCGCCCTGTGTACACGGATTCTG  
 HisAspGlyLeuAlaGluAspPheSerLeuTyrLeuHisAlaProCysValThrAspSer

4670 4680 4690 4700 4710 4720  
 TCACTGGCGCCTGAAGGTTGCGGCAGTTACTATGTGTTGGCGCCGGTGCCGCATTTAGGC  
 SerLeuAlaProGluGlyCysGlySerTyrTyrValLeuAlaProValProHisLeuGly

4730 4740 4750 4760 4770 4780  
 ACCGCGAACCTCGACTGGACGTTGAGGGGCCAAAACCTACGCGACCGTATTTTTGCGTAC  
 ThrAlaAsnLeuAspTrpThrValGluGlyProLysLeuArgAspArgIlePheAlaTyr

4790 4800 4810 4820 4830 4840  
 CTTGAGCAGCATTACATGCCTGGCTTACGGAGTCAGCTGGTCACGCACCGGATGTTTACG  
 LeuGluGlnHisTyrMetProGlyLeuArgSerGlnLeuValThrHisArgMetPheThr

4850 4860 4870 4880 4890 4900  
 CCGTTTGATTTTCGCGACCAGCTTAATGCCTATCATGGCTCAGCCTTTTCTGTGGAGCCC  
 ProPheAspPheArgAspGlnLeuAsnAlaTyrHisGlySerAlaPheSerValGluPro

FIG. 4 (b)

4910	4920	4930	4940	4950	4960
GTTCTTACCCAGAGCGCCTGGTTTCGGCCGCATAACCGCGATAAAACCATTACTAATCTC					
ValLeuThrGlnSerAlaTrpPheArgProHisAsnArgAspLysThrIleThrAsnLeu					
4970	4980	4990	5000	5010	5020
TACCTGGTCGGCGCAGGCACGCATCCCGGCGCAGGCATTCTGGCGTCATCGGCTCGGCA					
TyrLeuValGlyAlaGlyThrHisProGlyAlaGlyIleProGlyValIleGlySerAla					
5030	5040	5050	5060		
AAAGCGACAGCAGGTTTGATGCTGGAGGATCTGATTGGA					
LysAlaThrAlaGlyLeuMetLeuGluAspLeuIle***					
				↑	
				H	

FIG. 4 (c)

5100 5110 5120 5130 5140 5150  
ATGGCAGTTGGCTCGAAAAGTTTTCGACAGCCTCAAAGTTATTTGATGCAAAAACCCGG  
MetAlaValGlySerLysSerPheAlaThrAlaSerLysLeuPheAspAlaLysThrArg

5160 5170 5180 5190 5200 5210  
CGCAGCGTACTGATGCTCTACGCCTGGTGCCGCCATTGTGACGATGTTATTGACGATCAG  
ArgSerValLeuMetLeuTyrAlaTrpCysArgHisCysAspAspValIleAspAspGln

5220 5230 5240 5250 5260 5270  
ACGCTGGGCTTTCAGGCCCGGCAGCCTGCCTTACAAACGCCCGAACAACGTCTGATGCAA  
ThrLeuGlyPheGlnAlaArgGlnProAlaLeuGlnThrProGluGlnArgLeuMetGln

5280 5290 5300 5310 5320 5330  
CTTGAGATGAAAACGCGCCAGGCCTATGCAGGATCGCAGATGCACGAACCGGCGTTTGCG  
LeuGluMetLysThrArgGlnAlaTyrAlaGlySerGlnMetHisGluProAlaPheAla

5340 5350 5360 5370 5380 5390  
GCTTTTCAGGAAGTGGCTATGGCTCATGATATCGCCCCGGCTTACGCGTTTGATCATCTG  
AlaPheGlnGluValAlaMetAlaHisAspIleAlaProAlaTyrAlaPheAspHisLeu

5400 5410 5420 5430 5440 5450  
GAAGGCTTCGCCATGGATGTACGCGAAGCGCAATACAGCCAACTGGATGATACGCTGCGC  
GluGlyPheAlaMetAspValArgGluAlaGlnTyrSerGlnLeuAspAspThrLeuArg

5460 5470 5480 5490 5500 5510  
TATTGCTATCACGTTGCAGGCGTTGTGCGCTTGATGATGGCGCAAATCATGGGCGTGCGG  
TyrCysTyrHisValAlaGlyValValGlyLeuMetMetAlaGlnIleMetGlyValArg

5520 5530 5540 5550 5560 5570  
GATAACGCCACGCTGGACCGCGCCTGTGACCTTGGGCTGGCATTTCAGTTGACCAATATT  
AspAsnAlaThrLeuAspArgAlaCysAspLeuGlyLeuAlaPheGlnLeuThrAsnIle

5580 5590 5600 5610 5620 5630  
GCTCGCGATATTGTGGACGATGCGCATGCGGGCCGCTGTTATCTGCCGGCAAGCTGGCTG  
AlaArgAspIleValAspAspAlaHisAlaGlyArgCysTyrLeuProAlaSerTrpLeu

5640 5650 5660 5670 5680 5690  
GAGCATGAAGGTCTGAACAAAGAGAATTATGCGGCACCTGAAAACCGTCAGGCGCTGAGC  
GluHisGluGlyLeuAsnLysGluAsnTyrAlaAlaProGluAsnArgGlnAlaLeuSer

5700 5710 5720 5730 5740 5750  
CGTATCGCCCGTCGTTTGGTGCAGGAAGCAGAACCTTACTATTTGTCTGCCACAGCCGGC  
ArgIleAlaArgArgLeuValGlnGluAlaGluProTyrTyrLeuSerAlaThrAlaGly

FIG. 5 (a)

5760 5770 5780 5790 5800 5810  
CTGGCAGGGTTGCCCCTGCGTTCCGCCTGGGCAATCGCTACGGCGAAGCAGGTTTACCGG  
LeuAlaGlyLeuProLeuArgSerAlaTrpAlaIleAlaThrAlaLysGlnValTyrArg

5820 5830 5840 5850 5860 5870  
AAAATAGGTGTCAAAGTTGAACAGGCCGGTCAGCAAGCCTGGGATCAGCGGCAGTCAACG  
LysIleGlyValLysValGluGlnAlaGlyGlnGlnAlaTrpAspGlnArgGlnSerThr

5880 5890 5900 5910 5920 5930  
ACCACGCCCGAAAAATTAACGCTGCTGCTGGCCGCCTCTGGTCAGGCCCTTACTTCCCGG  
ThrThrProGluLysLeuThrLeuLeuLeuAlaAlaSerGlyGlnAlaLeuThrSerArg

5940 5950 5960 5970 5980  
ATGCGGGCTCATCCTCCCCGCCCTGCGCATCTCTGGCAGCGCCCGCTCTAG  
MetArgAlaHisProProArgProAlaHisLeuTrpGlnArgProLeu\*\*\*

↑  
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FIG. 5 (b)



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ATGTTGTGGATTGGAATGCCCTGATCGTTTTTCGTTACCGTGATTGGCATGGAAGTGATT  
MetLeuTrpIleTrpAsnAlaLeuIleValPheValThrValIleGlyMetGluValIle

↑  
K

GCTGCACTGGCACACAAATACATCATGCACGGCTGGGGTTGGGGATGGCATCTTTCACAT  
AlaAlaLeuAlaHisLysTyrIleMetHisGlyTrpGlyTrpGlyTrpHisLeuSerHis

CATGAACCGCGTAAAGGTGCGTTTGAAGTTAACGATCTTTATGCCGTGGTTTTTGCTGCA  
HisGluProArgLysGlyAlaPheGluValAsnAspLeuTyrAlaValValPheAlaAla

TTATCGATCCTGCTGATTTATCTGGGCAGTACAGGAATGTGGCCGCTCCAGTGGATTGGC  
LeuSerIleLeuLeuIleTyrLeuGlySerThrGlyMetTrpProLeuGlnTrpIleGly

GCAGGTATGACGGCGTATGGATTACTCTATTTTATGGTGCACGACGGGCTGGTGCATCAA  
AlaGlyMetThrAlaTyrGlyLeuLeuTyrPheMetValHisAspGlyLeuValHisGln

CGTTGGCCATTCCGCTATATTCCACGCAAGGGCTACCTCAAACGGTTGTATATGGCGCAC  
ArgTrpProPheArgTyrIleProArgLysGlyTyrLeuLysArgLeuTyrMetAlaHis

CGTATGCATCACGCCGTCAGGGGCAAAGAAGGTTGTGTTTCTTTTGGCTTCCTCTATGCG  
ArgMetHisHisAlaValArgGlyLysGluGlyCysValSerPheGlyPheLeuTyrAla

CCGCCCCTGTCAAACTTCAGGCGACGCTCCGGGAAAGACATGGCGCTAGAGCGGGCGCT  
ProProLeuSerLysLeuGlnAlaThrLeuArgGluArgHisGlyAlaArgAlaGlyAla

GCCAGAGATGCGCAGGGCGGGGAGGATGAGCCCGCATCCGGGAAGTAA  
AlaArgAspAlaGlnGlyGlyGluAspGluProAlaSerGlyLys\*\*\*

5925

↑  
L

FIG. 6

1	10	20	30	40	50
GGTACCGCAC	GGTCTGCCAA	TCCGACGGAG	GTTTATGAAT	TTTCCACCTT	TTCCACAAGC
70	80	90	100	110	
TCAACTAGTA	TTAACGATGT	GGATTTAGCA	AAAAAAACCT	GTAACCCTAA	ATGTAAAATA
130	140	150	160	170	
ACGGGTAAGC	CTGCCAACCA	TGTTATGGCA	GATTAAGCGT	CTTTTTGAAG	GGCACC GCAT
190	200	210	220	<sup>A</sup> 230	
CTTTCGCGTT	GCCGTAAATG	TATCCGTTTA	TAAGGACAGC	CCGAATGACG	GTCTGCGCAA
250	260	270	280	290	
AAAAACACGT	TCATCTCACT	CGCGATGCTG	CGGAGCAGTT	ACTGGCTGAT	ATTGATCGAC
310	320	330	340	350	
GCCTTGATCA	GTTATTGCCC	GTGGAGGGAG	AACGGGATGT	TGTGGGTGCC	GCGATGCGTG
370	380	390	400	410	
AAGGTGCGCT	GGCACC GGGA	AAACGTATTC	GCCCCATGTT	GCTGTTGCTG	ACCGCCCGCG
430	440	450	460	470	
ATCTGGGTTG	CGCTGTCAGC	CATGACGGAT	TACTGGATTT	GGCCTGTGCG	GTGGAAATGG
490	500	510	520	530	
TCCACGCGGC	TTGCTGATC	CTTGACGATA	TGCCCTGCAT	GGACGATGCG	AAGCTGCGGC
550	560	570	580	590	
GCGGACGCCC	TACCATT CAT	TCTCATTACG	GAGAGCATGT	GGCAATACTG	GCGGCGGTTG
610	620	630	640	650	
CCTTGCTGAG	TAAAGCCTTT	GGCGTAATTG	CCGATGCAGA	TGGCCTCAGC	CCGCTGGCAA
670	680	690	700	710	
AAAATCGGGC	GGTTTCTGAA	CTGTCAAACG	CCATCGGCAT	GCAAGGATTG	G TTCAGGGTC
730	740	750	760	770	
AGTTCAAGGA	TCTGTCTGAA	GGGGATAAGC	CGCGCAGCGC	TGAAGCTATT	TTGATGACGA
790	800	810	820	830	
ATCACTTTAA	AACCAGCACG	CTGTTTTGTG	CCTCCATGCA	GATGGCCTCG	ATTGTTGCGA
850	860	870	880	890	
ATGCCTCCAG	CGAAGCGCGT	GATTGCCTGC	ATCGTTTTTC	ACTTGATCTT	GGTCAGGCAT
910	920	930	940	950	
TTCAACTGCT	GGACGATTTG	ACCGATGGCA	TGACCGACAC	CGGTAAGGAT	AGCAATCAGG
970	980	990	1000	1010	
ACGCCGGTAA	ATCGACGCTG	GTCAATCTGT	TAGGCCCGAG	GGCGGTTGAA	GAACGTCTGA
1030	1040	1050	1060	1070	
GACAACATCT	TCAGCTTGCC	AGTGAGCATC	TCTCTGCGGC	CTGCCAACAC	GGGCACGCCA
1090	1100	1110	1120	1130 <sup>B</sup>	
CTCAACATTT	TATTCAGGCC	TGGTTTGACA	AAAAACTCGC	TGCCGTCAGT	TAAGGATGCT

FIG. 7 (a)

C  
 1150 1160 1170 1180 1190  
 GCATGAGCCA TTTCGCGGCG ATCGCACCGC CTTTTTACAG CCATGTTCGC GCATTACAGA  
 1210 1220 1230 1240 1250  
 ATCTCGCTCA GGAACCTGGTC GCGCGCGGTC ATCGGGTGAC CTTTATTTCAG CAATACGATA  
 1270 1280 1290 1300 1310  
 TTAAACACTT GATCGATAGC GAAACCATTG GATTTCATTG CGTCGGGACA GACAGCCATC  
 1330 1340 1350 1360 1370  
 CCCCCGGCGC GTTAACGCGC GTGCTACACC TGGCGGCTCA TCCTCTGGGG CCGTCAATGC  
 1390 1400 1410 1420 1430  
 TGAAGCTCAT CAATGAAATG GCGCGCACCA CCGATATGCT GTGCCGCGAA CTCCCCCAGG  
 1450 1460 1470 1480 1490  
 CATTTAACGA TCTGGCCGTC GATGGCGTCA TTGTTGATCA AATGGAACCG GCAGGCGCGC  
 1510 1520 1530 1540 1550  
 TCGTTGCTGA AGCACTGGGA CTGCCGTTTA TCTCTGTGCG CTGCGCGCTG CCTCTCAATC  
 1570 1580 1590 1600 1610  
 GTGAACCGGA TATGCCCCCTG GCGGTTATGC CTTTCGAATA CGGGACCAGC GACGCGGCTC  
 1630 1640 1650 1660 1670  
 GCGAACGTTA TGCCGCCAGT GAAAAAATTT ATGACTGGCT AATGCGTTCGT CATGACCGTG  
 1690 1700 1710 1720 1730  
 TCATTGCCGA ACACAGCCAC AGAATGGGCT TAGCCCCCGG GCAAAGCTT CACCAGTGTT  
 1750 1760 1770 1780 1790  
 TTTCGCCACT GGCGCAAATC AGCCAGCTTG TTCCTGAACT GGATTTTCCC CGCAAAGCGT  
 1810 1820 1830 1840 1850  
 TACCGGCTTG TTTTCATGCC GTCGGGCCTC TGC CGGAAAC GCACGCACCG TCAACGTCTT  
 1870 1880 1890 1900 1910  
 CATCCCGTTA TTTTACATCC TCAGAAAAAC CCCGATTTT CGCCTCGCTG GGCACGCTTC  
 1930 1940 1950 1960 1970  
 AGGGACACCG TTATGGGCTG TTAAAAACGA TAGTGAAAGC CTGTGAAGAA ATTGACGGTC  
 1990 2000 2010 2020 2030  
 AGCTCCTGTT AGCCCACTGT GGTCGTCTTA CGGACTCTCA GTGTGAAGAG CTGGCGCGAA  
 2050 2060 2070 2080 2090  
 GCCGTCATAC ACAGGTGGTG GATTTTGCCG ATCAGTCAGC CGCGCTGTCT CAGGCGCAGC  
 2110 2120 2130 2140 2150  
 TGGCGATCAC CCACGGCGGC ATGAATACGG TACTGGACGC GATTAATTAC CGGACGCCCC  
 2170 2180 2190 2200 2210  
 TTTTAGCGCT TCCGCTGGCC TTTGATCAGC CCGGCGTCGC GTCACGCATC GTTTATCACG  
 2230 2240 2250 2260 2270  
 GCATCGGCAA GCGTGCTTCC CGCTTTACCA CCAGCCATGC TTTGGCTCGT CAGATGCGTT

FIG. 7 (b)

2290	2300	2310	2320	2330
CATTGCTGAC	CAACGTCGAC	TTTCAGCAGC	GCATGGCGAA	AATCCAGACA
2350	2360	2370	2380	2390
TGGCAGGGGG	CACCATGGCC	GCTGCCGATA	TCATTGAGCA	GGTTATGTGC
2410	2420	2430	2440	2450
CTGTCTTAAG	TGGGAGCGGC	TATGCAACCG	CATTATGATC	TGATTCTCGT
2470	2480	2490	2500	2510
CTCGCGAATG	GCCTTATCGC	CCTGCGTCTT	CAGCAGCAGC	AACCTGATAT
2530	2540	2550	2560	2570
CTTATCGACG	CCGCACCCCA	GGCGGGCGGG	AATCATACGT	GGTCATTTCA
2590	2600	2610	2620	2630
TTGACTGAGA	GCCAACATCG	TTGGATAGCT	CCGCTGGTGG	TTCATCACTG
2650	2660	2670	2680	2690
CAGGTACGCT	TTCCACACG	CCGTCGTAAG	CTGAACAGCG	GCTACTTTTG
2710	2720	2730	2740	2750
CAGCGTTTCG	CTGAGGTTTT	ACAGCGACAG	TTTGGCCCCG	ACTTGTGGAT
2770	2780	2790	2800	2810
GTCGCAGAGG	TTAATGCGGA	ATCTGTTCGG	TTGAAAAGG	GTCAGGTTAT
2830	2840	2850	2860	2870
GCGGTGATTG	ACGGGCGGGG	TTATGCGGCA	AATTCAGCAC	TGAGCGTGGG
2890	2900	2910	2920	2930
TTTATTGGCC	AGGAATGGCG	ATTGAGCCAC	CCGCATGGTT	TATCGTCTCC
2950	2960	2970	2980	2990
GATGCCACGG	TCGATCAGCA	AAATGGTTAT	CGCTTCGTGT	ACAGCCTGCC
3010	3020	3030	3040	3050
ACCAGATTGT	TAATTGAAGA	CACGCACTAT	ATTGATAATG	CGACATTAGA
3070	3080	3090	3100	3110
GCGCGGCAAA	ATATTTGCGA	CTATGCCGCG	CAACAGGGTT	GGCAGCTTCA
3130	3140	3150	3160	3170
CGAGAAGAAC	AGGGCGCCTT	ACCCATTACT	CTGTCGGGCA	ATGCCGACGC
3190	3200	3210	3220	3230
CAGCGCCCCC	TGGCCTGTAG	TGGATTACGT	GCCGGTCTGT	TCCATCCTAC
3250	3260	3270	3280	3290
TCACTGCCGC	TGGCGGTTGC	CGTGGCCGAC	CGCCTGAGTG	CACTTGATGT
3310	3320	3330	3340	3350
GCCTCAATTC	ACCATGCCAT	TACGCATTTT	GCCCGCGAGC	GCTGGCAGCA
3370	3380	3390	3400	3410
TTCCGCATGC	TGAATCGCAT	GCTGTTTTTA	GCCGGACCCG	CCGATTACAG

FIG. 7 (c)

3430 3440 3450 3460 3470  
 ATGCAGCGTT TTTATGGTTT ACCTGAAGAT TTAATTGCCG GTTTTTATGC GGGAAAACCTC  
 3490 3500 3510 3520 3530  
 ACGCTGACCG ATCGGCTACG TATTCTGAGC GGCAAGCCGC CTGTTCCGGT ATTAGCAGCA  
 3550 3560 3570 3580 3590  
 TTGCAAGCCA TTATGACGAC TCATCGTTAA AGAGCGACTA CATGAAACCA ACTACGGTAA  
 3610 3620 3630 3640 3650  
 TTGGTGACAGG CTTCCGGTGGC CTGGCACTGG CAATTCGTCT ACAAGCTGCG GGGATCCCCG  
 3670 3680 3690 3700 3710  
 TCTTACTGCT TGAACAACGT GATAAACCCG GCGGTCGGGC TTATGTCTAC GAGGATCAGG  
 3730 3740 3750 3760 3770  
 GGTTTACCTT TGATGCAGGC CCGACGGTTA TCACCGATCC CAGTGCCATT GAAGAACTGT  
 3790 3800 3810 3820 3830  
 TTGCACTGGC AGGAAAACAG TTAAAAGAGT ATGTCGAACT GCTGCCGGTT ACGCCGTTTT  
 3850 3860 3870 3880 3890  
 ACCGCCTGTG TTGGGAGTCA GGGAAGGTCT TTAATTACGA TAACGATCAA ACCCGGCTCG  
 3910 3920 3930 3940 3950  
 AAGCGCAGAT TCAGCAGTTT AATCCCCGCG ATGTCGAAGG TTATCGTCAG TTTCTGGACT  
 3970 3980 3990 4000 4010  
 ATTCACGCGC GGTGTTTAAA GAAGGCTATC TAAAGCTCGG TACTGTCCCT TTTTATCGT  
 4030 4040 4050 4060 4070  
 TCAGAGACAT GCTTCGCGCC GCACCTCAAC TGGCGAAACT GCAGGCATGG AGAAGCGTTT  
 4090 4100 4110 4120 4130  
 ACAGTAAGGT TGCCAGTTAC ATCGAAGATG AACATCTGCG CCAGGCGTTT TCTTTCCACT  
 4150 4160 4170 4180 4190  
 CGCTGTTGGT GGGCGGCAAT CCCTTCGCCA CCTCATCCAT TTATACGTTG ATACACGCGC  
 4210 4220 4230 4240 4250  
 TGGAGCGTGA GTGGGGCGTC TGGTTTCCGC GTGGCGGCAC CGGCGCATTG GTTCAGGGGA  
 4270 4280 4290 4300 4310  
 TGATAAAGCT GTTTCAGGAT CTGGGTGGCG AAGTCGTGTT AAACGCCAGA GTCAGCCATA  
 4330 4340 4350 4360 4370  
 TGGAACGAC AGGAAACAAG ATTGAAGCCG TGCATTTAGA GGACGGTCGC AGGTTCCCTGA  
 4390 4400 4410 4420 4430  
 CGCAAGCCGT CGCGTCAAAT GCAGATGTGG TTCATACCTA TCGCGACCTG TTAAGCCAGC  
 4450 4460 4470 4480 4490  
 ACCCTGCCGC GGTAAAGCAG TCCAACAAAC TGCAGACTAA GCGCATGAGT AACTCTCTGT  
 4510 4520 4530 4540 4550  
 TTGTGCTCTA TTTTGGTTTG AATCACCATC ATGATCAGCT CGCGCATCAC ACGGTTTGTT

4570	4580	4590	4600	4610
TCGGCCCCGCG	TTACCGCGAG	CTGATTGACG	AAATTTTAA	TCATGATGGC
4630	4640	4650	4660	4670
ACTTCTCACT	TTATCTGCAC	GCGCCCTGTG	TCACGGATTC	GTCAGTGGCG
4690	4700	4710	4720	4730
GCGGCAGTTA	CTATGTGTTG	GCGCCGGTGC	CGCATTTAGG	CACCGCGAAC
4750	4760	4770	4780	4790
CGGTTGAGGG	GCCAAACTA	CGCGACCGTA	TTTTTGCGTA	CCTTGAGCAG
4810	4820	4830	4840	4850
CTGGCTTACG	GAGTCAGCTG	GTCACGCACC	GGATGTTTAC	GCCGTTTGAT
4870	4880	4890	4900	4910
AGCTTAATGC	CTATCATGGC	TCAGCCTTTT	CTGTGGAGCC	CGTTCTTACC
4930	4940	4950	4960	4970
GGTTTCGGCC	GCATAACCGC	GATAAAACCA	TTACTAATCT	CTACCTGGTC
4990	5000	5010	5020	5030
CGCATCCCGG	CGCAGGCATT	CCTGGCGTCA	TCGGCTCGGC	AAAAGCGACA
5050	<sup>H</sup> 5060	5070	5080	5090
TGCTGGAGGA	TCTGATTGGA	ATAATCCGTC	GTTACTCAAT	CATGCGGTCTG
5110	5120	5130	5140	5150
AGTTGGCTCG	AAAAGTTTTG	CGACAGCCTC	AAAGTTATTT	GATGCAAAAA
5170	5180	5190	5200	5210
CGTACTGATG	CTCTACGCCT	GGTGCCGCCA	TTGTGACGAT	GTTATTGACG
5230	5240	5250	5260	5270
GGGCTTTCAG	GCCCCGGCAGC	CTGCCTTACA	AACGCCCGAA	CAACGTCTGA
5290	5300	5310	5320	5330
GATGAAAACG	CGCCAGGCCT	ATGCAGGATC	GCAGATGCAC	GAACCGGCGT
5350	5360	5370	5380	5390
TCAGGAAGTG	GCTATGGCTC	ATGATATCGC	CCCGGCTTAC	GCGTTTGATC
5410	5420	5430	5440	5450
CTTCGCCATG	GATGTACGCG	AAGCGCAATA	CAGCCAACCTG	GATGATACGC
5470	5480	5490	5500	5510
CTATCACGTT	GCAGGCGTTG	TCGGCTTGAT	GATGGCGCAA	ATCATGGGCG
5530	5540	5550	5560	5570
CGCCACGCTG	GACCGCGCCT	GTGACCTTGG	GCTGGCATTT	CAGTTGACCA
5590	5600	5610	5620	5630
CGATATTGTG	GACGATGCGC	ATGCGGGCCG	CTGTTATCTG	CCGGCAAGCT
5650	5660	5670	5680	5690
TGAAGGTCTG	AACAAAGAGA	ATTATGCGGC	ACCTGAAAAC	CGTCAGGCGC

FIG. 7 (e)

5710	5720	5730	5740	5750
CGCCCGTCGT	TTGGTGCAGG	AAGCAGAACC	TTACTATTTG	TCTGCCACAG
5770	5780	5790	5800	5810
AGGGTTGCCC	CTGCGTTCCG	CCTGGGCAAT	CGCTACGGCG	AAGCAGGTTT
5830	5840	5850	5860	5870
AGGTGTCAAA	GTTGAACAGG	CCGGTCAGCA	AGCCTGGGAT	CAGCGGCAGT
5890	5900	5910	5920	5930
GCCCGAAAAA	TTAACGCTGC	TGCTGGCCGC	CTCTGGTCAG	GCCCTTACTT
5950	5960	5970	5980	5990
GGCTCATCCT	CCCCGCCCTG	CGCATCTCTG	GCAGCGCCCG	CTCTAGCGCC
6010	6020	6030	6040	6050
CGGAGCGTCG	CCTGAAGTTT	TGACAGGGGC	GGCGCATAGA	GGAAGCCAAA
6070	6080	6090	6100	6110
CCTTCTTTGC	CCCTGACGGC	GTGATGCATA	CGGTGCGCCA	TATACAACCG
6130	6140	6150	6160	6170
CCCTTGCGTG	GAATATAGCG	GAATGGCCAA	CGTTGATGCA	CCAGCCCGTC
6190	6200	6210	6220	6230
AAATAGAGTA	ATCCATACGC	CGTCATACCT	GCGCCAATCC	ACTGGAGCGG
6250	6260	6270	6280	6290
GTACTGCCCA	GATAAATCAG	CAGGATCGAT	AATGCAGCAA	AAACCACGGC
6310	6320	6330	6340	6350
TTAACTTCAA	ACGCACCTTT	ACGCGGTTCA	TGATGTGAAA	GATGCCATCC
6370	6380	6390	6400	6410
CCGTGCATGA	TGTATTTGTG	TGCCAGTGCA	GCAATCACTT	CCATGCCAAT
6430	6440	6450	6460	6470
AAAACGATCA	GGGCATTCCA	AATCCACAAC	ATAATTTCTC	CGGTAGAGAC
6490	6500	6510	6520	6530
AGGCTTAAGG	ATTCAATTTT	AACAGAGATT	AGCCGATCTG	GCGGCGGGAA
6550	6560	6570	6580	6590
CGCGCCAGAA	AGGCGCGCCA	GGGATCAGAA	GTCGGCTTTC	AGAACCACAC
6610	6620	6630	6640	6650
TTTACCTGCA	CGAACATGGT	CCAGTGCATC	GTTGATTTTC	GACATCGGGA
6670	6680	6690	6700	6710
TGTCGGCGCA	ATATCTGTAC	GGCCAGCCAG	CTTCAGCAGT	GAACGCAGCT
6730	6740	6750	6760	6770
ACCGGTTGAA	GAACCCGTCA	CGGCGCGGTC	GCCTAAAATC	AGGCTGAAAG
6790	6800	6810	6820	6830
CAAACGGCTT	CAGTACGGCA	CCCACGGTAT	GGAACCTACC	GCGAGGCGCC

FIG. 7 (f)

6850 6860 6870 6880 6890  
AGTAGGGTTG CCAGTCGAGA TCGACGGCGA CCGTGCTGAT AATCAGGTCA AACTGGCCCG  
6910 6918  
CCAGGCTTTT TAAAGCTT

FIG. 7 (g)



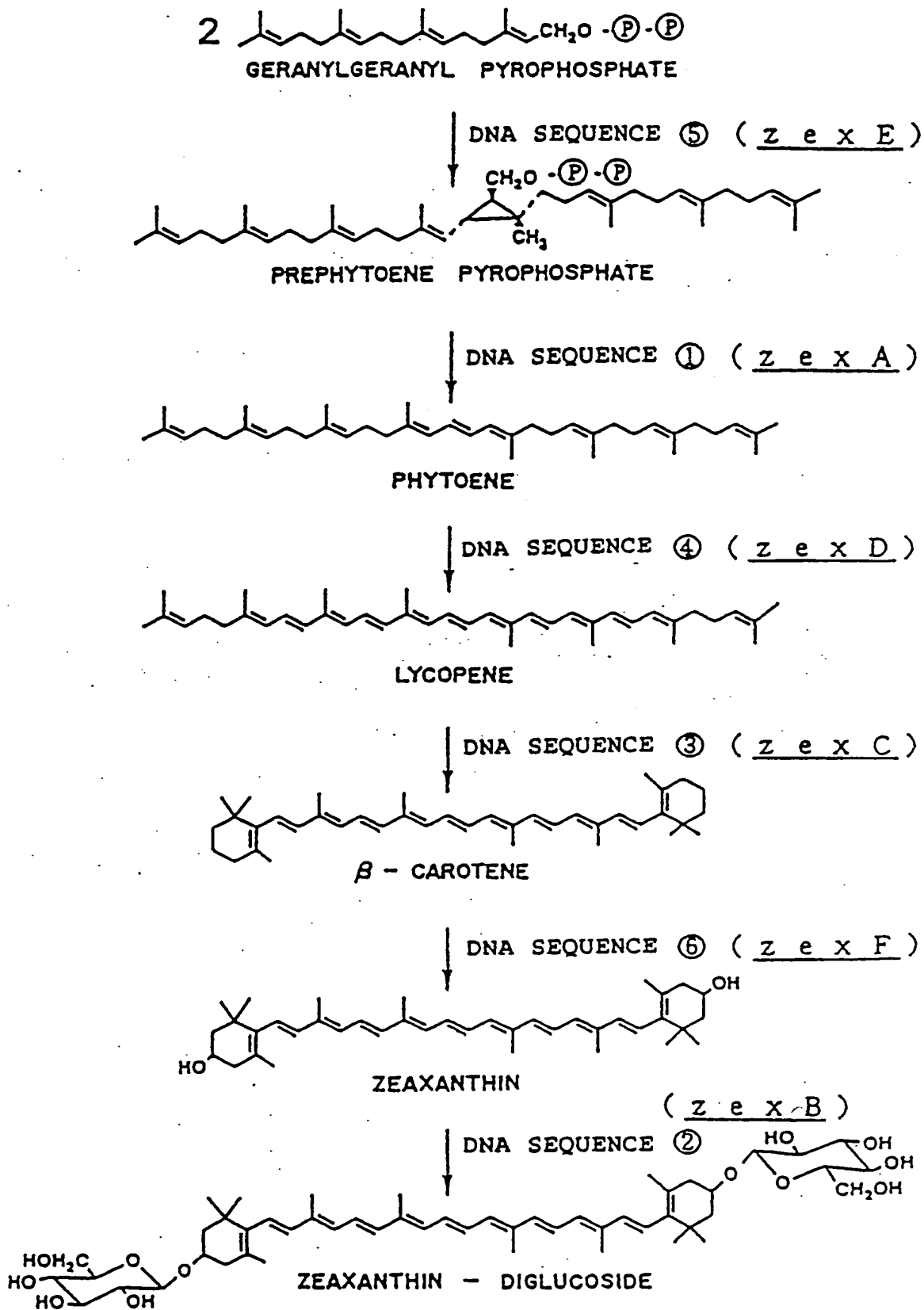


FIG. 8



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 90 10 7493

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	JOURNAL OF BACTERIOLOGY, vol. 168, no. 2, November 1986, pages 607-612, American Society for Microbiology; K.L. PERRY et al.: "Cloning and regulation of Erwinia herbicola pigment genes" ---		C 12 N 15/52 C 12 N 9/00 C 12 P 23/00 C 12 P 9/00 C 12 P 5/00
A	J. GEN. MICROBIOL., vol. 130, 1984, pages 1623-1631, SGM; G. THIRY: "Plasmids of the epiphytic bacterium Erwinia uredovora" ---		
A	CHEMICAL ABSTRACTS, vol. 109, no. 17, 24th October 1988, page 172, abstract no. 143512y, Columbus, Ohio, US; G. GIULIANO et al.: "A genetic-physical map of the Rhodobacter capsulatus carotenoid biosynthesis gene cluster", & MGG, MOL. GEN. GENET. 1988, 213(1), 78-83 ---		
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 10-07-1990	Examiner DESCAMPS J.A.
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document  T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 5)
A	CHEMICAL ABSTRACTS, vol. 107, no. 7, 17th August 1987, page 222, abstract no. 53404u, Columbus, Ohio, US; J.M. PEMBERTON et al.: "Expression of Rhodopseudomonas sphaeroides carotenoid photopigment genes in phylogenetically related nonphotosynthetic bacteria", & CURR. MICROBIOL. 1987, 15(2), 67-71 -----		
			TECHNICAL FIELDS SEARCHED (Int. Cl. 5)
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